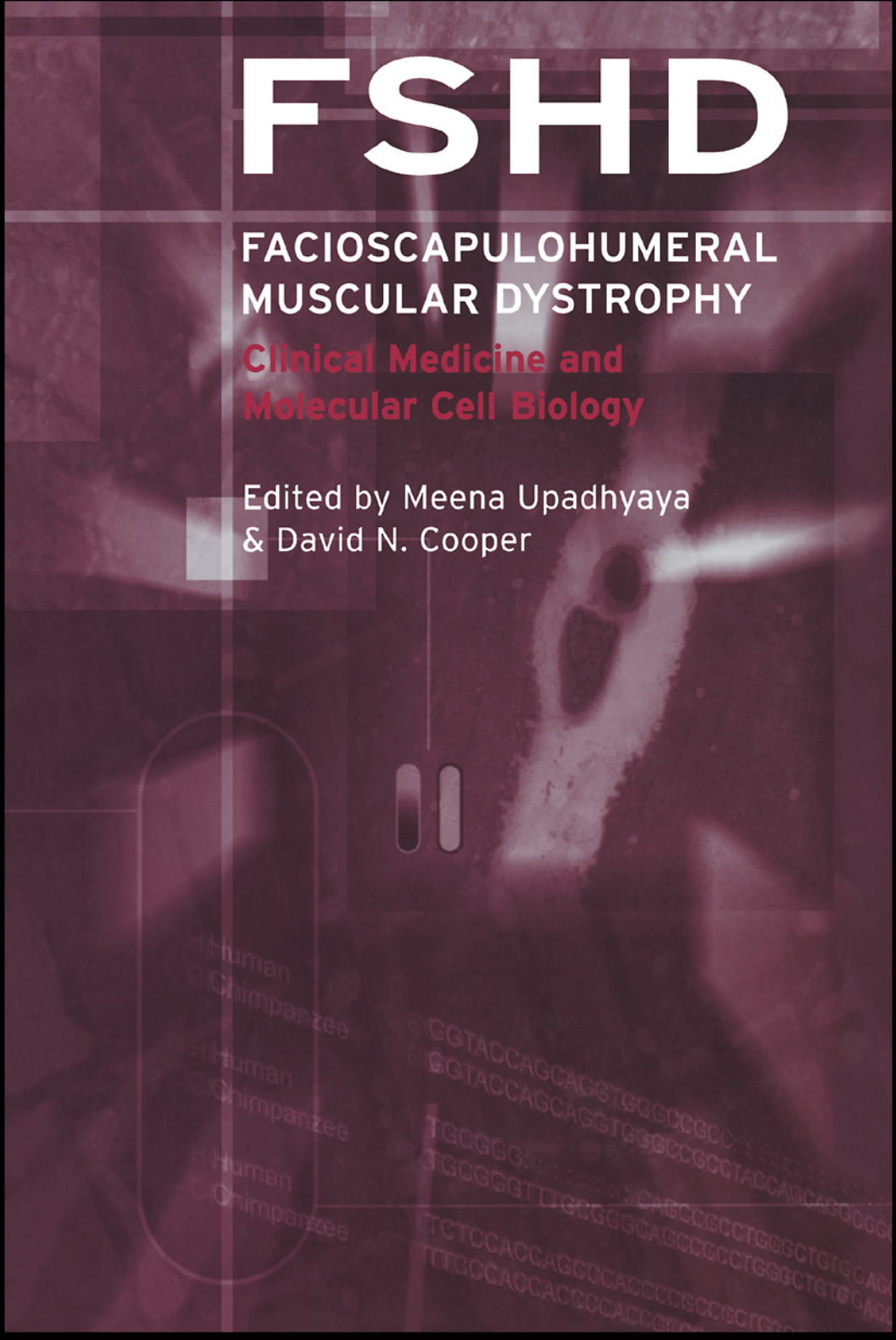

FSHD Facioscapulohumeral Muscular Dystrophy

Clinical Medicine and Molecular Cell Biology

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Clinical Medicine and Molecular Cell Biology

Edited by

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Contents

Abbreviations

ALS	amyotrophic lateral sclerosis
BLAST	basic local alignment search tool
BMD	Becker muscular dystrophy
cdk	cyclin-dependent kinase
cDNA	complementary DNA
CK	creatine kinase
DMD	Duchenne muscular dystrophy
DFC	dense fibrillar component
ECG	electrocardiogram
EDMD	Emery-Dreifuss muscular dystrophy
EMSA	electrophoretic mobility shift assay
EST	expressed sequence tag
FC	fibrillar centre
FGF	fibroblast growth factor
FIGE	field inversion gel electrophoresis
FISH	fluorescent *in situ* hybridization
FSHD	facioscapulohumeral muscular dystrophy
FSLD	facioscapulolimb dystrophy
GC	genetic counselling
GC	granular component
HMG	high mobility group
IEF	isoelectric focusing
LCR	locus control region
LGMD	limb girdle muscular dystrophy
MHC	myosin heavy chain
MRF	myogenic regulatory factor
MRI	magnetic resonance imaging
NF1/2	neurofibromatosis type 1/2

NLS	nuclear localization signal
ORF	open reading frame
PBL	peripheral blood lymphocyte
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
PEV	position effect variegation
PFGE	pulsed-field gel electrophoresis
PRE	polycomb response element
PTPC	permeability transition pore complex
RFLP	restriction fragment length polymorphism
RT	reverse transcription
SCARMD	severe childhood autosomal recessive muscular dystrophy
SMA	spinal muscular atrophy
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
snRNP	small nuclear ribonuclear protein
SPECT	single-photon-emission computed tomography
SSCP	single-strand conformational polymorphism
STS	sequence tagged site
TRE	trithorax response element
VNTR	variable number tandem repeat

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Dedication

To Rachna and the memory of Krishna.

Foreword

Peter S.Harper

Facioscapulohumeral muscular dystrophy (FSHD) is both a fascinating and a challenging disorder for researchers as well as for clinicians. As one of the most frequent muscular dystrophies of adult life, it has long deserved a book specifically devoted to it; there is far too much important information to fit readily into a single chapter or chapter section of a more general book on muscle disease, as has been the case until now. So this book on FSHD is both welcome and necessary, since without a single comprehensive source, it is difficult for those involved in the disorder to obtain a clear and balanced account of its different aspects.

Clinicians and clinical geneticists will find this a source of real practical value, especially when faced with difficult diagnostic dilemmas and complex genetic counselling situations. Laboratory scientists will also find the clinical sections helpful, often throwing light on aspects of more basic work that would otherwise seem puzzling.

For those involved in FSHD research, this book brings together all the main strands of current work, with the key workers across the world all contributing to it. The basic mechanisms underlying the condition are still elusive, but promise to make fundamental contributions to biology, as has already occurred with a range of other neuromuscular disorders.

The FSHD research community is an active, collaborative and innovative one, and there are still many challenges ahead for it. So it is of the greatest value to have this book to give a full and clear picture of our current knowledge, as a platform on which future research can be based. Meena Upadhyaya, deeply involved in FSHD research for many years, and her close colleague David Cooper, are to be congratulated in providing us with a synthesis that will not only be of practical help to clinicians and scientists, but should also encourage other workers to contribute to the still unsolved challenges for our fuller understanding of this important condition.

1.
Introduction and overview of FSHD

Meena Upadhyaya, and David N.Cooper

FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

1.1 Introduction

FSHD is a unique genetic disorder. For a long time, the molecular basis of this condition was enigmatic. Indeed, some 13 years elasped between the mapping of the chromosomal location of the gene and deducing the nature of the unique molecular mechanism underlying the disease. The last decade has thus been an extremely exciting one for FSHD research *(Figure 1.1)*. During this time it is clear that there has been a very significant increase in our knowledge of the molecular genetics of FSHD.

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common inherited neuromuscular condition, after Duchenne and myotonic muscular dystrophies (Upadhyaya and Cooper, 2002). FSHD is an autosomal dominant inherited disorder characterized by progressive muscle weakness and involving atrophy of the muscles of the face, upper arm and shoulder girdle (see Chapters 2 and 3). General muscle weakness and atrophy may eventually involve musculature of the pelvic girdle and the foot extensor. Early onset of the disease is usually associated with the development of the most severe forms of the disorder (Lunt and Harper, 1991; Padberg, 1998). Disease onset is unusual before the age of 10, however, being observed in fewer than 5% of patients, in whom it is associated with significant facial weakness. The majority of patients only develop symptoms later during the second decade of life. Both retinal vasculopathy and high-tone deafness may be seen as part of FSHD (see Chapter 13). However, clinically significant deafness is rare in adult FSHD. In some families, the affected individuals share many of the clinical features of FSHD but show no evidence of facial muscle involvement, even in the most severely affected patients. These ‘scapulohumeral muscular dystrophy’ families display an autosomal dominant mode of inheritance, and it has been suggested that they may form part of the FSHD disease spectrum (Jardine *et al.*, 1994; Tawil *et al.*, 1995; Felice *et al.*, 2000). In some FSHD families, the disease is associated with mental retardation and epilepsy (see Chapter 14).

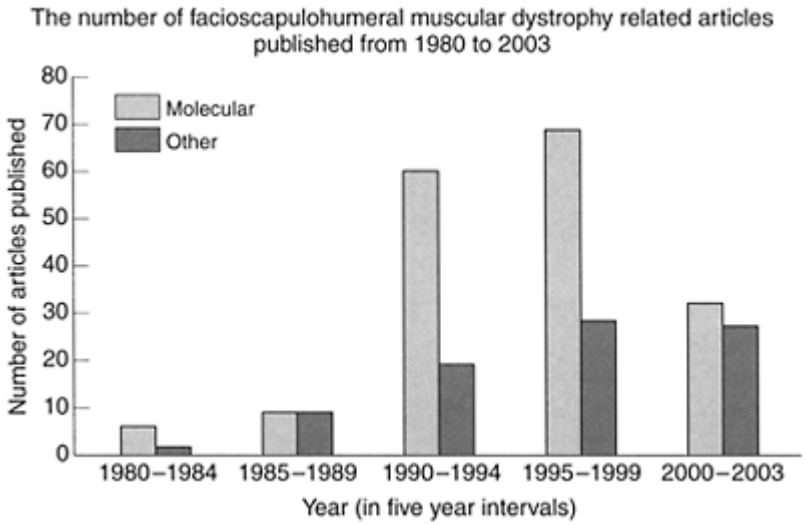

Figure 1.1 The total annual number of publications relating to FSHD retrieved from PubMed (NCBI), from 1980.

1.2 Gene mapping studies

The FSHD locus was genetically mapped to chromosome 4q35 (Upadhyaya *et al.*, 1990; Wijmenga *et al.*, 1990) where it is closely linked to the complex locus D4F104S1 *(Figure 1.2)* identified by Southern analysis using probe p13E-11 (Wijmenga *et al.*, 1992). Details of the mapping studies are given in Chapter 4. There is some evidence for genetic heterogeneity in FSHD. Although the vast majority of large FSHD families can be demonstrably linked to 4q35, a small number of classical FSHD families have been excluded from this location (Gilbert *et al.*, 1993). A genomic position for this second disease locus, tentatively designated *FSHD1B*, has still to be identified (see Chapter 19).

1.2.1 DNA rearrangements associated with D4F104S1 and D4Z4 repeats

Probe p13E-11 (D4F104S1) identifies an *Eco*RI restriction fragment, with homology to both 4q35 and 10q26. In FSHD patients, one fragment derived from one of the two homologous chromosomes 4, is usually smaller than 35 kb. Studies in suitably informative FSHD families have demonstrated the 4q35 component of the D4F104S1 locus to be the closest marker to the disease gene. Sequence analysis of the entire D4F104S1 locus has shown that p13E-11 identifies a single copy genomic sequence that is located immediately proximal to an array of tandem repeat units. It is variation in the exact number of individual repeats, in each of the 4q35- and 10q26-derived repeat arrays,

that constitutes the polymorphic element of the D4F104S1 variable number tandem repeat (VNTR) and of the two specific *Eco*RI restriction sites which define this VNTR; one site is located proximal to the sequence homologous to the p13E-11 probe, whereas the second is situated immediately distal to the tandem repeat array (see *Figure 1.2)*.

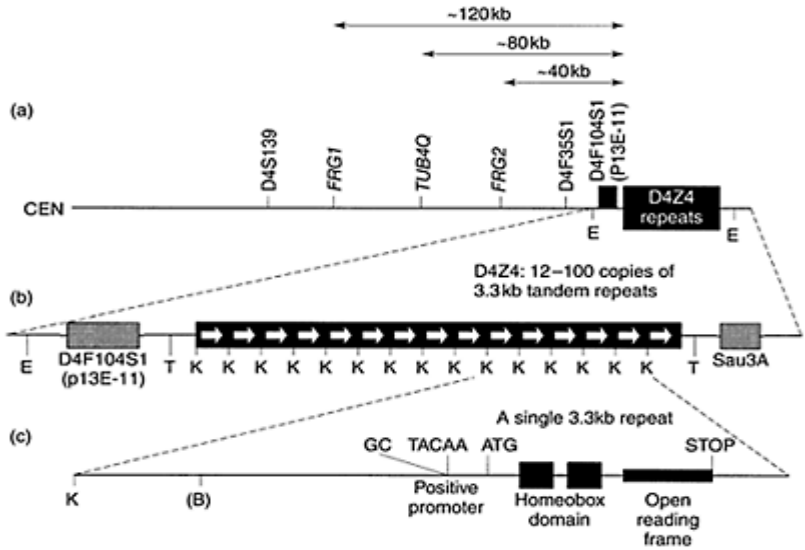

Figure 1.2 Restriction map of the FSHD candidate region at 4q35: Relative positions of *FRG1*, *TUBB4Q*, *FRG2*, D4F104S1 (p13E11) and D4Z4. The *Eco*RI fragment detected by probe p13E11 predominantly comprises an array of 3.3 kb tandem repeats that have a copy number of 12–100 in normal controls and usually <10 in FSHD patients. Each 3.3 kb repeat comprises two homeodomains (shaded boxes) encompassing an ORF with an in-frame start codon (ATG) and a stop codon. It also encodes the *DUX4* gene. The position of the GC and TACAA boxes in the promoter-like sequence of *DUX4* gene is indicated. B: *Bln*I restriction site, E: *Eco*RI restriction

site; K: *Kpn*I restriction site; T: *Tru*91 restriction site.

Each (virtually identical?) repeat unit is 3.3 kb in size and has been designated D4Z4, regardless of whether the repeat is located within the 4q35- or the 10q26-derived repeat array. The sizes of the variable fragments seen on Southern blots, containing *Eco*RI-digested high-molecular-weight genomic DNA derived from normal individuals and hybridized with probe p13E-11, range from ~35–38 kb to more than 300 kb whereas in FSHD patients, they range from 7 to 35–38 kb (see Chapter 4). Since it is difficult to resolve fragment sizes over 50 kb with conventional Southern blots, pulsed-field gel electrophoresis (PFGE) has been employed to increase resolution. Since the majority of people carry two copies of each homologous chromosome, there is, in an informative situation, the potential to observe four differently sized *Eco*RI fragments when the DNA from one individual is analysed. Each fragment represents a different D4F104S1 allele, with two alleles being derived from the 4q35 locus and two alleles from the 10q26 locus (both loci produce *Eco*RI fragments with the same size range). Following such analyses in a number of FSHD families, it was soon realized that the size of at least one of the four D4F104S1 alleles seen in the affected members of these families, differed significantly from the normal size range: patients possessed *Eco*RI fragments in the range from ~35 kb down to ~10 kb.

This potential disease association was confirmed and strengthened by demonstrating the *de novo* occurrence of a smaller *Eco*RI fragment in the majority of sporadic FSHD patients studied, that was not present in either of the clinically unaffected parents. A small proportion of FSHD patients, of both sporadic and familial origin, do not exhibit this *Eco*RI fragment, either because (i) such cases arise via a different mutational mechanism, (ii) they represent families unlinked to the *FSHD1A* locus at 4q35, or (iii) they are phenocopies of other conditions. What pathognomonic process could turn this apparently normal variation into a diseasecausing mutation? One clue is perhaps to be found in the observation that the severity of disease expression in FSHD patients correlates with the size of their disease-associated D4F104S1 alleles. Thus, patients with the smallest *Eco*RI fragments manifest the severe form of the disorder and present with an earlier age of onset (Lunt *et al.*, 1995).

If the possession of one short D4F104S1 allele is sufficient to confer the disease phenotype (FSHD is a dominant disorder, and hence, only requires a change in one of the two copies of the *FSHD* gene), then one might infer that the complete loss of one of the D4F104S1 alleles would yield a still more severe phenotype. A number of individuals have been identified who carry small cytogenetically detectable deletions involving specifically the 4q region and, as a result, are monosomic for one copy of their 4q35-derived D4F104S1 loci. Surprisingly, none of these individuals showed any evidence of an FSHD-like disorder; indeed, most of them appeared to be phenotypically normal. These findings indicated that FSHD most probably results from a dominant negative (‘gain-of-function’) mutational mechanism rather than the loss or partial loss of a gene product (haploinsufficiency) from within the 4q-deleted region. These observations served to focus the search for the *FSHD* gene back onto the D4F104S1 locus itself, and in particular to the D4Z4 repeats therein.

1.2.2 D4Z4 repeats

The D4Z4 repeat units are members of a large family of 3.3 kb tandem repeat loci that are located on the short arm of the acrocentic chromosomes, the pericentromeric regions (especially on chromosome 1), and the telomeric regions of the long arms of chromosomes 4 and 10 (Hewitt *et al.,* 1994; Lyle *et al.,* 1995). Chapter 6 describes the structural organization and evolution of the D4Z4 repeats. The size variation observed at the D4F104S1 locus is caused by the loss of individual discrete D4Z4 repeat units and does not involve internal deletions within the body of the repeat itself (van Deutekom *et al.,* 1993). Thus, the normal size range of the D4F104S1 alleles, from about 38 kb to more than 300 kb, represents D4Z4 repeat arrays containing from 11–100 repeats. By contrast, the FSHD-associated D4F104S1 allele size range, from 10 kb to ~38 kb, represents arrays containing between one and 11 D4Z4 repeat units. Large-scale physical mapping and sequencing studies of the entire D4F104S1 genomic region have demonstrated: (i) that the 4q35 D4Z4 arrays are located immediately adjacent to the 4q telomere; and (ii) that a copy of a single D4Z4 repeat-like sequence is located about 30 kb proximal to the p13E-1 1 homologous sequence in the opposite orientation to the D4Z4 repeats in the distally located tandem arrays.

1.2.3 Sequence homology and genetic recombination between 4q35 and 10q26

Various studies have shown that a duplicate (virtually identical) copy of the D4F104S1 locus is located at 10q26, within the heterochromatic subtelomeric region of chromosome 10 (Deidda *et al.,* 1995). Detailed sequence analysis of D4Z4 repeats from both the 4q35 and 10q26 D4F104S1 loci confirmed their high level of sequence homology (98–100%) and, perhaps more importantly, identified a unique *Bln*I restriction site that is present within each copy of the 10q26-derived repeat units, but which is absent from the 4q35-derived D4Z4 repeats (Deidda *et al.,* 1996).

The high degree of sequence homology between the 4q35 and 10q26 D4F104S1 regions may be responsible for the observed ‘interchromosomal exchanges’ between these two loci (see Chapter 7). During these subtelomeric exchanges, complete repeat arrays (either of 4q35-derived *Bln*I-resistant D4Z4 repeats, or of 10q26-derived *Bln*I-sensitive repeats) are ‘transferred’ from one chromosomal location to the other. Owing to the sizes of the genomic fragments involved, these interchromosomal exchange events are best visualized with PFGE; such studies have shown that entire repeat arrays are ‘translocated’ in the majority of studied cases (van Deutekom *et al.,* 1996a). It should however be stated that there is no direct evidence for the actual physical translocation of genetic material and interchromosomal gene conversion would perhaps appear to be a more plausible mechanism.

Perhaps surprisingly, these dynamic subtelomeric interchanges do not appear to be involved in any way with expression of the FSHD phenotype. Such 4q35 10q26 exchanges are evident in about 20% of normal individuals. It should be emphasized, though, that FSHD only occurs when the D4Z4 repeats that are deleted (regardless of whether they are originally chromosome 4q35- or 10q26-derived) are chromosome 4 located (van Deutekom *et al.,* 1996a). Recently, it has been demonstrated that in FSHD patients, homogeneous chromosome 10-specific repeats are rarely encountered. Rather,

these repeats are invariably combined with chromosome 4-specific repeats (van Overveld *et al.,* 2000). The molecular mechanism(s) underlying these subtelomeric exchanges has still to be elucidated, as has their potential influence (if any) on FSHD disease expression.

1.3 Somatic mosaicism

Somatic mutations that occur sufficiently early on in embryonic life involve both somatic cells and the germline. These individuals (gonosomal mosaics) may also be at risk of having affected children. Often a new mutation first appears in mosaic form, usually in a clinically normal person, who then has a constitutionally affected child. Somatic mosaicism in FSHD has been reported (Upadhyaya *et al.,* 1995; Kohler *et al.,* 1996). van der Maarel *et al.* (2000) detected somatic mosaicism in 40% of *de novo* FSHD families (14% in an unaffected parent and 26% of the *de novo* FSHD patients themselves). Interestingly, an excess of mosaic-affected males was found in this dataset. Chapter 12 describes mosaicism in FSHD.

1.4 The genotype/phenotype relationship in FSHD

An association between the size of the deleted *Eco*RI fragment and the age at disease onset (smaller *Eco*RI fragments are always associated with the most severe form of the disease) has been observed in patients from a number of different ethnic groups (Lunt *et al.,* 1995; Zatz *et al.,* 1995; Tawil *et al.,* 1996; Hsu *et al.,* 1997). The D4F104S1 *Eco*RI fragment size range noted in severe childhood cases is 10–18 kb, in typical teenage-onset cases between 18–34 kb, and in the oldest late-onset patients larger than 30 kb (Lunt *et al.,* 1995; see Chapter 11). Two recent Japanese studies have confirmed these observations; a number of very-early-onset patients, with a severe form of FSHD often accompanied by epilepsy and mental retardation, manifested short *Eco*RI/*Bln*I fragments, the smallest being 10 kb in length (Funakoshi *et al.,* 1998; Muira *et al.,* 1998). Chapter 14 gives an account of unusual clinical features in FSHD. Genotype/phenotype correlations do not however always appear to be sustained in the borderline region of 8–14 D4Z4 repeats (Butz *et al.,* 2003).

It is important to stress that it is not yet possible to predict accurately the likely severity of disease expression in any one individual. This is due to the high degree of inter- and intrafamilial variability of disease expression observed in this disorder, regardless of the fact that all affected members of a family exhibit the same size of D4F104S1 allele. As an example, the clinical picture presented by a typical three-generation FSHD family can be used to illustrate the spectrum of clinical variability often observed between affected individuals from the same family. In this family, the grandfather was only very mildly affected whereas the father was moderately affected. The grandchild, the propositus, was the most severely affected and had been confined to a wheelchair from the age of 10 years. Molecular diagnosis established that an identically sized 18 kb *Eco*RI/*Bln*I fragment was present in all three affected members of this family (Upadhyaya *et al.,* 1999). In addition to the fragment size, mosaicism also contributes significantly to the severity of the disease.

The correlation between D4Z4 repeat number and clinical severity has been supported by recent findings that illustrate that a multiprotein complex (YY1, HMGB2, nucleolin) binds to D4Z4 and serves to reduce the expression of the 4q35 genes *FRG1, FRG2* and *ANT1*. D4Z4 deletion results in the pathological overexpression of these genes (Gabellini *et al.,* 2002). Furthermore, the level of overexpression was inversely correlated to the number of D4Z4 repeats both *in vivo* and *in vitro*, indicating that D4Z4 repeat number is critical in regulating the transcriptional misregulation that results in the FSHD phenotype.

1.5 Gender bias

There appears to be a gender-specific influence on the degree and rate of disease progression in FSHD. The age of disease onset is invariably later in female gene carriers who are also more likely to exhibit a less severe form of the disease (Zatz *et al.*, 1998). It has been suggested that female hormonal status somehow confers a mild protective effect. Consistent with this view, disease progression is markedly accelerated in female patients following the menopause, at which time a general decline in muscle strength becomes apparent. Gender bias in FSHD is discussed in Chapter 20.

1.6 Molecular diagnosis of FSHD

As we have seen, FSHD is associated with partial deletions of the polymorphic D4Z4 repeat array on 4q35. This deletion is visualized in *Eco*RI-digested DNA by hybridization with probe p13E-11 (see Chapter 15 for a review of molecular diagnosis). Usage of differential *Eco*RI/*Bln*I double digests led to a dramatic improvement in the application of a molecular diagnostic approach for FSHD (Bakker *et al.*, 1996; Upadhyaya *et al.*, 1997). Although the specificity and sensitivity of this *Eco*RI-*Bln*I double-digest technique for accurate FSHD diagnosis has been assessed and demonstrated to be high, an additional diagnostic complication is created by subtelomeric chromosomal exchanges between the two highly homologous 4q35 and 10q26 D4Z4 repeat loci which are present in about 20% of the normal population (van Deutekom *et al.*, 1996a). Diagnostic complexity has been further increased by the recent observation that these subtelomeric exchanges may also result in the formation of hybrid arrays, containing an admixture of 4q35- and 10q26-derived D4Z4 repeat units (van der Maarel *et al.*, 1999). In addition, the complete loss of the D4F104S1 locus (probe p13E-11) has been reported in FSHD patients (Lemmers *et al.*, 2003). Subtelomeric exchanges may lead to problems during the molecular diagnosis of FSHD in 5% of cases (Bakker *et al.*, 1996; van Deutekom *et al.*, 1996a). Recent findings utilizing an additional restriction enzyme (*Xap*I) have increased the accuracy of molecular diagnosis for FSHD to 98% (Lemmers et al., 2001).

A number of factors have however served to complicate the molecular diagnosis of FSHD:

1. The high level of sequence homology between the FSHD-associated region at 4q35 and other chromosome regions.
2. The complex dynamics of 4q35 and 10q26 interchromosomal exchanges.

3. Evidence of locus heterogeneity in FSHD families.
4. A complex disease mechanism: a few FSHD patients from 4q35-linked families do not manifest a small disease-associated D4F104S1 *Eco*RI/*Bln*I allele.
5. The high incidence of somatic and germline mosaicism.

Although molecular diagnosis for FSHD can now be offered with up to 98% accuracy, there is still a need to improve the basis of this, since it is still based on the use of Southern analysis. With technological advances, however, the vast majority of molecular tests are now PCR-based and usually permit a result to be generated within hours rather than the 7–10 days required for the traditional FSHD test. There is, however, an urgent need for the development of a simple, reliable test which does not require the use of PFGE.

Although a relationship between mutant genotype and clinical phenotype has been reported in FSHD patients, it is still not possible to predict the likely severity of disease expression in any one individual owing to the high intrafamilial variability of disease expression observed. This is despite the presence of the identically deleted D4F104S1 alleles in all affected family members. Genetic counselling for FSHD patients is discussed in Chapter 21.

1.7 FSHD muscle biochemistry and physiology

Relatively few structural studies of FSHD muscle tissue have been performed. The ultrastructural, immunocytochemical, molecular and protein characteristics of FSHD muscle are discussed in Chapter 18. The sarcolemmal aspects are described in Chapter 22 and reveal that the organization of the sarcolemma of FSHD muscle is altered and that the closeness of its links to the underlying contractile apparatus is compromised. In addition, the response of FSHD myoblasts to oxidative stress is presented in Chapter 16. Myoblasts were subjected to oxidative stress using varying concentrations of paraquat, a generator of superoxide anions. FSHD myoblasts were more vulnerable to paraquat than control myoblasts for lower concentration of paraquat. In addition, FSHD myoblasts have reduced replicative capacity and a morphological appearance resembling senescence.

1.8 The search for the *FSHD* gene

The search for the elusive *FSHD* gene is described in Chapters 5 and 9. Following on from the recognition of the close association between large D4F104S1 locus deletions and disease expression, it was anticipated that the *FSHD* gene would be quickly identified and characterized. However, despite extensive efforts worldwide, it has not been possible to pinpoint the *FSHD* gene. Although the D4F104S1 deletions are closely associated with FSHD in some way, the identity and location of the *FSHD* gene (or genes?) still remains elusive.

The D4Z4 repeat at 4q35 has been shown to include the *DUX4* gene (Gabriels *et al.*, 1999). Initial attempts to demonstrate that the homeobox-like sequences associated with each D4Z4 repeat are functional have been somewhat equivocal. In this volume, Coppee *et al.* (Chapter 9) show that the DUX4 protein is expressed in myoblasts of patients with

FSHD but not in normal muscle. They have identified a similar *DUX4C* gene located in a truncated and inverted member of the 3.3 kb D4Z4 repeat family 50 kb centromeric to the D4Z4 locus.

The search for the *FSHD* gene has been focused upon the genomic region located proximal to the repeat arrays. Extensive sequence analysis and the application of the basic local alignment search tool (BLAST) to this genomic region was undertaken to search for possible genes and the locations of any expressed sequences identified in Expressed Sequence Tag (EST) database screens. This genomic region was found: (i) to be enriched in several types of repetitive element, and (ii) to contain a number of gene-like sequences homologous to loci dispersed across the genome, and which appear to represent pseudogenes (van Deutekom *et al.,* 1995). The highly repetitive nature of the 4q35 region has seriously hampered the search for a possible FSHD candidate gene; particularly difficult has been the identification of suitable single-copy DNA probes required for use in many hybridization-based gene identification methods, such as cDNA selection. Several successful exon trapping and cDNA library screens have been carried out with specific genomic subclones, but results have generally been disappointing. The close proximity of the FSHD region to the 4q telomere has probably also hindered many cloning studies; similar subtelomeric regions are known to contain few functional genes (Flint *et al.,* 1996). Another possible problem could be if the *FSHD* gene was normally to function only at some specific point during development; such a developmental stage-specific gene might only be transiently expressed, and then only in certain tissues. Thus, cDNA libraries screened to date may either simply not contain the *FSHD* gene transcript, or it has been present at too low a level to be detected by the relatively insensitive gene selection methodologies so far employed.

1.9 Potential gene sequences within the FSHD candidate region

The 161 kb genomic region situated proximal to the D4Z4 repeats has been sequenced and the sequence analysed with a number of gene prediction programs (van Geel *et al.,* 1999). Almost half (45%) of this subtelomeric region is made up of repeat sequences, of which LINE-1 elements form the bulk (see Chapter 8). Several retrotransposed genes have also been identified in this genomic region (van Geel *et al.*, 1999). In addition to the known genes and pseudogenes from the region (*FRG1, FRG2* and *TUBB4Q*), a number of potential coding regions were identified, although none of these potential gene sequences has been confirmed by RT-PCR or cDNA library screening. Further studies are clearly required to assess the functionality or otherwise of these potential coding regions. The genomic analysis of 10q and 4q telomeres and their relevance to FSHD is discussed in Chapter 8.

A polymorphism in the B-satellite repeats located distal to the D4Z4 repeat has recently been identified. This polymorphism, comprising alleles 4qA and 4qB, occurs with nearly equal frequencies in the normal population. FSHD is however uniquely associated with allele 4qA allele (Lemmers *et al.,* 2002; van Geel *et al.,* 2002). Allele 4qA may have additional features which give rise to FSHD either by facilitating the derepression consequent to D4Z4 deletion or conceivably by promoting D4Z4 deletion mutagenesis directly. It remains to be defined how allele 4qA relates to the complex that

controls the expression of genes on 4q35. This finding may lead to a better understanding of the instability identified in the FSHD region.

1.10 Methylation studies

It has been assumed that D4Z4 arrays are normally heterochromatic and that this heterochromatinization in unaffected individuals may spread from the D4Z4 repeats to the proximally located genes. In support of this postulate, DNA within the D4Z4 repeats in normal subjects has been shown to be hypermethylated (Tsien *et al.,* 2001) and these repeats contain hypoacetylated histones normally associated with the transcriptionally silent portion of the chromosome. Many D4Z4 CpG methylation-sensitive restriction sites are significantly hypomethylated in FSHD patients as compared to normal controls (van Overveld *et al.,* 2003). This finding was also observed in FSHD patients unlinked to 4q35, suggesting that hypomethylation may play an important role in FSHD. It has been proposed that normal heterochromatinization at 4q35 is lost when the number of D4Z4 repeats is reduced. The loss of heterochromatin spreading then results in an inappropriate increase in the expression of critical genes in *cis* in affected skeletal muscle. Chapter 17 explores hypotheses relating to the molecular aetiology of FSHD in relation to the loss of heterochromatin spreading and other long-range interaction models.

1.11 Expression studies in the FSHD candidate region

In the search for clues as to possible underlying molecular mechanisms responsible for FSHD, a recent study compared mRNA expression patterns present in skeletal muscle from FSHD patients and normal controls (Tupler *et al.,* 1999). Using an RNA subtraction hybridization technique to identify differentially expressed transcripts, FSHD dystrophic muscle was found to exhibit marked alterations in gene expression levels, revealing evidence of the significant underexpression or overexpression of a number of specific gene transcripts. Intriguingly, several of the abnormally expressed genes are known to be transcriptional regulators. It may be that the deregulated expression of such factors is reflected in the wide spectrum of genes that appear to be abnormally transcribed in FSHD muscle.

Gabellini *et al.* (2002) have recently demonstrated that a complex of proteins (YY1, HMGB2 and nucleolin) binds to a 27 bp DNA sequence in each D4Z4 repeat and that this binding is partially lost in FSHD patients. This complex serves to repress the expression of genes located proximal to the repeat array. Deletion of D4Z4 thus leads to the inappropriate transcriptional derepression of 4q35-located genes in muscle resulting in disease. The precise function of the three genes comprising the repression complex is not yet known (see Chapter 10).

Expression profile studies of FSHD muscle are extensively covered in Chapter 23. Several genes dysregulated in FSHD muscle are specifically involved in myogenesis, cellular differentiation and cell cycle control. These genes include *MLP,* a positive regulator of myogenesis; *DLK1,* involved in differentiation; *WEE1,* a Cdkinhibitory kinase; *TFRC,* involved in myoblast proliferation; *VLDLR* and *NDN,* both involved in

differentiation; *IGLL2,* involved in the immune response; *TXNIP,* mediates oxidative stress and finally *MT* (metallothionein), involved in cell growth, repair and differentiation (Winokur *et al.,* 2003). Finally, gene expression profiling experiments indicated that FSHD may result from the inappropriate triggering of myogenic gene transcription prior to the normal onset of differentiation (Winokur *et al.,* 2003).

1.12 Therapy

Currently there is no specific treatment available for FSHD. Therapy may involve surgery such as scapular fixation and tendon transfers. In a preliminary 3-month trial, albuterol, a ß2-agonist that normally increases muscle mass and causes satellite cells to proliferate, has provided some encouraging evidence of improvements to both muscle strength and mass (Kissel, 1998). Therapeutic trials and management of FSHD are covered in Chapter 24.

1.13 Possible disease mechanism

The failure of extensive searches to identify a definitive FSHD gene within the 4q35 candidate region, coupled with the direct association observed between the degree of D4Z4 repeat loss and the severity of FSHD disease expression, has resulted in investigators proposing a number of different pathognomonic mechanisms. For a considerable time it was believed that the underlying mutational mechanism involved a positional effect (Fisher and Upadhyaya, 1997). This was defined as a deleterious change in the level of specific gene expression resulting from a change in the genomic position of that gene relative to its normal chromosomal environment but not associated with an intragenic mutation or deletions. The chromosomal rearrangements could in principle alter the euchromatic structure by inducing much tighter chromatin packaging as found in heterochromatin. D4Z4 repeats physically separate the 4q telomere-associated heterochromatin from the more proximally located euchromatic gene-rich regions of 4q35. In FSHD patients, the extensive loss of D4Z4 repeats would place the proximal gene-rich region immediately adjacent to the 4q telomere which is predicted to inactivate these genes. However, since haploinsufficiency of the 4q35 region does not cause FSHD (Tupler *et al.,* 1996), this scenario is hard to explain. In addition, besides *ANT1,* the expression level of the none of the single copy genes at 4q35 is altered in FSHD muscle when compared to normal muscle (see Chapter 23).

1.14 Transcriptional derepression

A 27 bp element within D4Z4 that specifically binds a multiprotein complex consisting of YY1, HMGB2 and nucleolin, *in vitro* and *in vivo,* has been shown to mediate the transcriptional repression of genes at 4q35 (Gabellini *et al.,* 2002; see Chapter 10). YY1 is a complex protein that is involved in repressing and activating a number of gene promoters. HMGB2, a member of the family of high mobility group (HMG) proteins,

interacts with the minor groove of DNA thereby bending the DNA fragment to which it is bound; it is therefore considered to have an architectural function. Nucleolin, located in the nucleolus, is involved in chromatin structure formation, rRNA transcription and maturation, ribosome assembly and nucleocytoplasmic transport. Antisense experiments designed to decrease the intracellular levels of this 27 bp component have demonstrated that the reduction of YY1, HMGB2 or nucleolin results in the overexpression of the 4q35-located *FRG2* gene which is not expressed in normal cells and tissues (Gabellini *et al.*, 2003).

In FSHD patients who possess fewer D4Z4 repeats, the amount of suppressor is greatly reduced and therefore the disease may be seen as being caused by the derepression of genes proximal to the repeats. The deletion of repeated 27 kb elements in the subtelomeric region of 4q may act in *cis* on tissue-specific neighbouring genes by derepressing their transcriptional activity, thereby initiating a cascade of events that eventually leads to FSHD. This finding accounts for the fact that monosomy of 4q35 does not result in FSHD (Tupler *et al.*, 1996). This elegant work has unravelled a putative new molecular mechanism for FSHD that may well also be found to underlie other genetic conditions.

1.15 The FSHD enigma

Does FSHD represent an inherited disease with a unique pathogenetic mechanism? Although there is some evidence for genetic heterogeneity in FSHD, the vast majority of cases involve a mutational mechanism associated with 4q35. Unfortunately, the 4q35 genomic region containing the FSHD locus is not yet fully characterized. It is still unclear why FSHD is exclusively associated with the contracted allele carried on 4qA-bearing chromosomes and never on 4qB-bearing chromosomes. It is conceivable that protein binding to ß-satellite repeats on 4qA facilitate the deletion of D4Z4 repeats, resulting in the derepression of 4q35 genes in FSHD muscle. Any hypothesis that attempts to explain the underlying disease mechanism must take into account the high sequence complexity of this region, with its highly repetitive nature and its sequence homologies to loci on other chromosomes.

A number of hitherto puzzling observations also need to be considered when proposing a pathogenetic mechanism that encompasses all features of FSHD:

- Monosomy of the 4q35 region does not produce disease (Tupler *et al.*, 1996).
- Large deletions of the 10q26-located D4Z4 repeat arrays do not result in any discernible disease phenotype despite the almost identical sequence of the 4q35- and 10q26-located open reading frames (ORFs).
- Interchromosomal exchanges occur between chromosome 4- and 10-located repeats both in affected and normal individuals.
- Somatic mosaicism (ranging from normal to disease size range) has been observed in both asymptomatic parents and in affected individuals.
- There is an inverse correlation between chromosome 4-located D4Z4 repeat copy number and clinical severity.
- Variable clinical expression both between and within affected families.

- The clinical phenotype in monozygotic twins can be discordant (Tawil *et al.*, 1993; Tupler *et al.*, 1998).
- Progression of the disease is assymmetrical.
- There is significant hypomethylation of D4Z4 CpG methylation-sensitive restriction sites in FSHD.
- There is complete association of the 4qA polymorphic allele with the FSHD D4Z4 deletion.
- Abnormalities of gene expression are evident in FSHD muscle.
- The myogenesis of FSHD muscle is aberrant.
- FSHD myoblasts are more susceptible to oxidative stress.
- Derepression of the transcriptional activity of a number of genes is apparent in FSHD muscle.
- D4Z4 deletions may exert an effect on the expression of multiple genes.

As yet, the overall pathophysiology of FSHD is not fully understood and the exact nature of the underlying molecular and biochemical defects remain unknown (Fisher and Upadhyaya, 1997). Despite the absence of any detectable genetic instability or molecular variation within families, it is evident that the D4Z4 repeats are directly related to the severity of disease expression, even though the precise nature of the underlying disease mechanism remains to be elucidated. Once the underlying pathology is known, appropriate therapies may be devised for this enigmatic disorder.

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2.
Facioscapulohumeral muscular dystrophy: historical background and literature review

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2.1 Historical aspects

The earliest recognizable descriptions of facioscapulohumeral muscular dystrophy were made in the second half of the 19th century. The written accounts of presentations, given to the continental scientific establishments of the day, make fascinating reading. These give a clear idea of the historical context and understanding of neuromuscular disorders at that time.

Up to the middle of the 1800s, almost all progressive muscle disease was believed to be due to underlying primary neurological causes, and particularly atrophy of the anterior horn cells, causing what we would nowadays recognize as spinal muscular atrophy (SMA). In December 1851, Meryon presented his findings to the Royal Medical and Chirurgical Society clearly depicting the key elements of a primary muscle disease affecting eight boys in three families (Meryon, 1852). This is regarded by many as the first clear account of Duchenne muscular dystrophy (Emery, 1987a). Although Guillaume Duchenne de Boulogne, had been, by some accounts, almost 'obsessed' with muscle disease for many years, it was another 17 years before he presented his seminal work on the condition that now bears his name (Duchenne, 1868). He was intrigued by the clinical feature of pseudohypertrophy, seen in a subset of childhood-onset *'paralysie'*. He reported on his own studies on the electromyographic differences and cited the postmortem findings of Eulenberg-Cohnheim (1865) who had demonstrated muscle atrophy without atrophy of the nervous system, leading Duchenne to conclude the condition to be a primary muscle disorder. These ideas were outlined in his *'Recherches sur la paralysie musculaire pseudo-hypertrophique ou paralysie myo-sderosique'* (*Duchenne,* 1868). He also outlined other postmortem findings of Cruveilheirs who in 1852 described his observations *'…sur la paralysie musculaire atrophique'*. Cruveilheirs described extreme atrophy of the anterior spinal cord in an adult with proximal weakness,

and later facial and lingual involvement. Duchenne quoted this case in recognition of the fact that facial and lingual involvement occurred late in the neurogenic atrophies. However, as noted by Padberg, he failed to comment on a second patient described by Cruveilheirs—an 18-year-old man who had died from smallpox in 1848 (Padberg, 1982). This man had a severe facioscapulohumeral syndrome but complete sparing of the central, spinal and peripheral nervous systems. This unfortunate young man, therefore, almost certainly represents the first description of FSHD in the medical literature. Duchenne included in the differential diagnosis of *pseudo-hypertrophie myo-sclerosique* a clear description of an FSH syndrome.

While Duchenne was concentrating on pseudohypertrophy, two other French physicians, Landouzy and Déjérine, had been following another family. Their observations *'de la myopathie atrophique progressive, myopathie hereditaire debutant dans l'enfance par la face, sans alteration du systeme nerveux'* were presented by the head of their *laboratoire*, Vulpian, to the *Academie des Sciences* (Landouzy and Déjérine, 1884). As indicated by the title, they clearly recognized the key features of FSHD—a progressive wasting disorder, starting with the face in childhood, hereditary, without involvement of the nervous system. This commonly cited account, given by Vulpian, concentrated on the family whom Landouzy and Déjérine had been following for 11 years. But details of the brothers were actually first published by Landouzy in the *Memoires de la Société de Biologie* in 1874, followed by a third and lengthy (113 pages) follow-up account with Déjérine in 1885 (Landouzy, 1874; Landouzy and Déjérine, 1885). In these, Landouzy and Déjérine stated that 'the atrophy of the muscles of the face started at 3 years of age, giving him a characteristic facies', described by them as looking 'wide-eyed and stupid, with prominent lips'. As personally observed by them 'from the age of eighteen little by little the atrophy reached the upper limbs, then all the other muscles of the body… There was no paralysis and no sensory disturbance'. They noted the hereditary nature of FSHD since the father, brother and sister of the proband were affected; thus one family exhibited male-to-male transmission and an affected female, thereby excluding X-linked and recessive inheritance. They also hinted at the clinical variability of FSHD noting that father, brother and sister only had facial involvement. This man also died at a young age *'a la tuberculose pulmonaire'*. Histological examination by Landouzy and Déjérine (1884) showed 'simple atrophy of the primary muscle fascicles' and 'absolutely normal intramuscular nerves, nerves to the muscle of the face and the limbs. And likewise the anterior roots and the facial trunk'. This fairly brief presentation contains all the key elements of FSHD. It is, therefore, only reasonable that although Cruveilheirs may have been the first to describe a case of FSHD, since Landouzy and Déjérine were the first to recognize the clinical distinction of this disorder, that their names should be attached to the common eponymous alternative Landouzy-Déjérine disease (also occasionally called Duchenne-Landouzy-Déjérine disease). The year after Vulpian's presentation, Landouzy and Déjérine presented the much longer paper including remarkable drawings and photographs of the two brothers, Leon and George, seen 11 years apart in 1873 and 1884 (Landouzy and Déjérine, 1885). Although primarily a discussion on FSHD, the arguments used to distinguish this from *l'atrophie musculaire progressive de l'enfance* (Duchenne muscular dystrophy) and, for the modern reader, the somewhat confusingly named *l'atrophie musculaire progressive de l'adulte*,

type Aran-Duchenne (SMA) formed an important development into a detailed classification of these neuromuscular disorders which included:

1. Primarie myopathie progressive

i. Atrophie musculaire progressive de l'enfance (Duchenne)
ii. Type facio-scapulo-huméral (Landouzy et Déjérine)
iii. Type scapulo-huméral (Zimmerlin)
iv. Type fémoro-tibial (Eichorst)

2. Myélopathiques

i. Type Aran-Duchenne de Charcot
ii. Type scapulo-huméral de Vulpian.

I have not included the full classification but it is interesting to note that this is the first reference to the 'facio-scapulo-humerar' distribution of FSHD. It is also possibly the first reference to the atrophie musculaire de Duchenne (as such). In addition it recognizes a primary scapulohumeral myopathy. Like Duchenne, they clearly recognized the significance and distinction between the *myopathique* and *myélopathique* forms of *atrophie musculaire* described but not recognized by Cruveilhiers (Landouzy and Déjérine, 1885, p. 322). They, therefore, also included in the differential diagnosis the myelopathies with a scapulohumeral distribution.

Landouzy and Déjérine did not include genetic inheritance as part of the defining classification of these disorders. Amongst the myopathies, this did not come until 1911 when Eulenberg and Cohn first, and then Weitz in 1921, recognized the occurrence of autosomal dominant, recessive and X-linked recessive modes of inheritance (quoted by Tyler and Stephens, 1950, and Padberg, 1982). Difficulties over classification occurred over the following 20–30 years. In an attempt to further classify the myopathies, Bell (1942) examined the literature and clinical records from the National Hospital, Queen Square, London. She used inheritance in her classification whilst the main defining clinical criteria depended on the presence or absence of pseudohypertrophy or facial weakness. FSHD fell into group 3. However, probably because FSHD was regarded as a benign disorder, no cases of FSHD had been seen at the National Hospital within the 14-year study period and therefore she was reliant on the literature for all her cases. No clear mode of inheritance emerged. Debate continued regarding the importance of facial weakness, and with it the argument over recessive or dominant inheritance. This issue was tackled by Tyler and Stephens (1950) who examined 273 individuals, the at-risk offspring of a kindred involving 1249 individuals; '130 were affected and 143 normal' leading them to conclude that FSHD displayed 'a typical Mendelian dominant pattern of inheritance'. This is an enormously useful study for examining penetrance, and the clinical diversity of FSHD. Although they discussed other cases from the literature, since it is based on a single large family, it cannot be used to define the inheritance of all FSHD. The debate seems to have been settled shortly thereafter following Walton and Nattrass' article on the classification, natural history and treatment of the myopathies (1954). Although they had only personally examined 18 cases from four families, they were quite clear that FSHD was 'usually inherited in an autosomal dominant manner and only occasionally as a recessive trait'.

More recently, Padberg (1982) examined 190 cases of FSHD from 19 families. He defined FSHD as an autosomal dominant disorder. He further recognized the existence of sporadic cases, but because 'autosomal recessive disorders closely resembling FSHD have not been properly documented' did not feel there was sufficient evidence to support other modes of inheritance. Padberg also used the data on his families to examine gene penetrance, clinical variability, survival, genetic fitness, prevalence and laboratory investigations such as muscle enzymes, electromyography and muscle biopsy features. This period also represented the beginnings of genetic linkage analysis. Using ten of his families, Padberg *et al.* analysed 35 early markers but obtained no clear linkage with a maximum LOD score of 1.4 on chromosome 14q32 (Padberg *et al.,* 1984). An international consortium later excluded 95% of the genome but was still unable to locate the gene (Lunt, 1989). Subsequently, Wijmenga *et al.* (1990,1991) using first RFLP markers, and then microsatelite polymorphisms, localized the gene for FSHD to chromosome 4 and then to 4q, with further refinement of the locus by Upadhyaya *et al.* (1990). Approaches to identify the FSHD gene were however, unsuccessful, although several candidates were excluded (Wijmenga, 1993; Wijmenga *et al.,* 1993). Shortly after this, Hewitt *et al.* (1994) isolated the cosmid clone 13E, which mapped distal to all previous clones and was therefore the most telomeric available for study. After extensive subcloning by Wijmenga and Hewitt, these authors isolated an almost single copy probe p13-E11. Analysis of the ten Dutch families of Padberg and six sporadic cases of FSHD identified shorter p13E-11 fragments which co-segregated with the disease in families, and had arisen *de novo* in five of six sporadic cases (Wijmenga *et al.,* 1992a, 1992b). The p13E-11 shortened fragments were shown by van Deutekom *et al.* (1993) to contain multiple copies of a 3.2 kb tandem repeat array, D4Z4. Analysis of the tandem repeat showed that each repeat unit contained two homeobox genes (Hewitt *et al.,* 1994; Lee *et al.,* 1995a). Sequencing did not however reveal any mutation and RNA studies suggested that these genes were inactive in both patients and controls (Lee *et al.,* 1995b).

2.2 Clinical aspects

2.2.1 Distribution of affected muscles

There are three particularly characteristic features about the distribution of muscle involvement in FSHD: firstly the early involvement of the face; secondly, the involvement of the upper limb girdle before the lower; and thirdly, the at-times striking asymmetry of muscle involvement.

Facial involvement

From the very first descriptions by Landouzy (1874) and Landouzy and Déjérine (1885), it was clear that FSHD was a 'progressive muscular atrophy that starts in childhood with the face' producing a *'facies particulier'*, which was apparent from 3 years of age in their patient. As Walton and Nattrass (1954) point out, however, as many as two-thirds of patients are completely unaware of facial weakness. FSHD is not the only condition to affect the face, but Landouzy and Déjérine were the first to use the term *myopathic*

facies. In myotonic dystrophy the distribution of involved muscles is quite different (Harper, 2001). In other myopathies, facial involvement is usually a later feature and often, but not invariably, less severe (Walton and Nattrass, 1954; Ricker and Mertens, 1968; van der Kooi *et al.,* 1996a). The most obviously affected facial muscles are the orbicularis oris and orbicularis oculi. The extrinsic muscles of the eye are virtually never involved. Difficulty closing the eyes leads to patients sleeping with their eyes open, a feature often first noted by a partner or parent. This may be asymptomatic for many years but tends to lead to dry, uncomfortable, gritty eyes in the morning. More severe weakness may in rare cases lead to an exposure keratitis and corneal scarring (Padberg *et al.*, 1995a), but this can usually be prevented by appropriate intervention, including surgical insertion of gold weights and lateral tarsorrhaphy (Sansone *et al.*, 1997). Awake, the most obvious features are a slightly hyperthyroid staring appearance but without proptosis. In particular, the limbus of the eye is often visible, especially the inferior sector, which should normally be covered by the lower lid, leaving the white of the sclera clearly visible. In even mild weakness, blinking is often abnormal, patients tending to roll the eye superiorly as they blink—Bell's phenomenon. Involvement of the mouth produces the characteristic thick lips described by Landouzy and Déjérine. The lower lip tends to be the more prominent and both together somewhat protuberant described by some as *'bouche de tapir'* (Padberg, 1982; Brooke, 1986). The patient is unable to form the tight kiss, or pucker, caused by maximal contraction of this circular perilabial muscle. As a result, most patients are unable to whistle, suck, or blow up balloons. Often they have never appreciated the link between these mild inconveniences and the later development of shoulder weakness. In most series, 81–96% of cases will have facial weakness (Becker, 1953; Chung and Morton, 1959; Padberg, 1982; Lunt and Harper, 1991). These studies tend to select out scapulohumeral or scapuloperoneal families, some of whom have now been shown to be allelic to FSHD, and so may overestimate the frequency of facial involvement (Jardine *et al.,* 1994a). Even so, most clinicians recognize that not all patients will develop facial weakness, and the diagnostic criteria produced following a European Neuromuscular Centre workshop allow 50% of individuals within a family not to have facial involvement (Padberg *et al.*, 1992).

Involvement of the orbiculi is very characteristic but other muscles most commonly involved include the buccinators and the levator anguli oris. The temporalis and masseter muscles in contrast to myotonic dystrophy are, however, usually spared. Like the limbs, asymmetric facial involvement is not unusual, being most evident on smiling. Occasionally it can be so marked as to produce an apparent unilateral facial palsy but careful examination of the less severe side usually reveals weakness here as well. Most commonly, the asymmetry will involve the whole ipsilateral side but occasionally one side will be weaker around the mouth and the contralateral side around the eye.

Upper limb involvement

Involvement of the face occurs at an early age but since the majority of patients are unaware of their weakness, it is often only noticed if particularly looked for. Many years later the patient notices difficulty abducting the arms. The main effects of this are to cause difficulty in putting objects onto shelves above shoulder height, difficulty in combing hair, and easy fatigue. At this stage, the involvement of the muscle of the

shoulder can be so specific that the patient has little or no difficulty with any other activity. The chief muscles involved are the latissimus dorsi, serratus anterior, pectorals, subscapularis, rhomboids, trapezius, supraspinatus and infraspinatus, and deltoid. Tyler and Stephens (1950) examined 240 people with FSHD showing diagrammatically the common order of muscle progression. Of the above muscles, it is worth noting several particular features. The upper fibres of trapezius tend to be spared until very late in the course of the disease—contributing to the superior movement of the winged scapula. Anteriorly, the clavicular head of the pectoralis major is one of the first muscles to be involved producing a localized atrophy and additional typical axillary fold pointing towards the manubrium sterni. Likewise, involvement of the deltoid is also characteristically localized to the proximal fibres, producing an at-times strikingly visible shelf where the distal muscle is relatively spared. All three features are particularly characteristic of, although not exclusive to, FSHD and as with all muscle involvement in FSHD, may be markedly asymmetric in distribution.

Lower limb involvement

At this point it is worth re-emphasizing that the majority of FSHD patients present with involvement of the upper limb first. However, in a minority of patients, presentation may be with weakness of the lower limb. When this occurs it is almost always due to involvement of the peroneal compartment, presenting with a foot-drop. Not surprisingly such cases cause greater diagnostic difficulty especially if they are *de novo*, and/or there is no facial involvement. A not unusual first diagnosis is therefore that of scapuloperoneal dystrophy or less commonly scapuloperoneal atrophy. These patients do have involvement of the shoulder girdle but it may be overlooked if the unaware falls short of a full examination once the pelvic girdle is found to be normal.

To return to the more typical presentation, following involvement of the shoulder girdle, there are two common modes of progression. In both, weakness is likely to spread simultaneously down the arm to involve the biceps, triceps and brachioradialis. At about the same stage, in the lower limb, a little more commonly, involvement of the pelvic girdle is first noted (Becker, 1953; Walton and Nattrass, 1954; Chung and Morton, 1959). Also fairly commonly, the pelvis may be spared and the weakness may ‘jump’ or ‘skip’ to involve the peroneal muscles first (Tyler and Stephens, 1950; Chyatte *et al.*, 1966; Kazakov *et al.*, 1974; Padberg, 1982). It is an important diagnostic feature that involvement of the upper limb girdle is virtually always earlier and more severe than the lower limb girdle. This rule is not absolute but is sufficiently reliable to cause serious questioning of the diagnosis if pelvic girdle involvement occurs first. As indicated in the preceding paragraph, foot-drop may occur relatively early in the course of FSHD, but this should not be confused with early pelvic girdle involvement which is very rare. When lower limb weakness has started in the pelvis, spread to the knee flexors and extensors, then to the ankle dorsiflexors, is most usual. Where the peroneal muscles have been involved first, spread is usually to the pelvic girdle next, thence to the knees.

So far the progression of FSHD has been described as if it were inevitable that all FSHD patients go on to develop a relentless progressive dystrophy involving all muscles of the limbs. That is not entirely true. Many patients appear to have long static phases, where progression is so slow that they appear to be in a plateau phase. Most authors

would agree, however, that FSHD does not have true plateau phases, but rather that progression in some patients decelerates to such an extent that it appears to almost stop. For many patients there is, therefore, no further apparent progression, and 30% remain only mildly affected throughout life (Lunt and Harper, 1991). Thus in mild cases, there may only be very slight facial weakness, only noted by an inability to whistle, and mild weakness of shoulder abduction. More typically there is slow but just-perceptible deterioration over 20–30 years. Although FSHD is often regarded as a benign, mild condition (Bell, 1942; Walton and Nattrass, 1954), Lunt and Harper (1991) noted that 19% of heterozygotes will be wheelchair-bound by the age of 40 years. This figure increases further with increasing age. For these individuals, FSHD certainly is not a mild condition and even though it does not lead to a premature death for the majority, there is no doubt that disability can be considerable, especially when the issue of pain is also considered (Bushby *et al.*, 1998). In severe cases the dystrophy can spread to involve the forearm and even the intrinsic muscles of the hands (Becker, 1953). In these cases, disability is considerable, but even so, many patients remain remarkably stoical in the face of enormous practical difficulties with day-to-day activities.

Respiratory involvement

Even in severely affected patients, respiratory involvement is rare. Certainly there does not appear to be a major issue with swallowing difficulties or failure to expectorate as is seen in some other dystrophies. Assessment of pulmonary function can be difficult because of the great difficulty forming a decent seal around most devices used for measuring airflow, and is therefore frequently inaccurate. However, in extremely severe cases undoubtedly respiratory failure may occur. In some this may be secondary to skeletal complications (more likely due to rare cases of scoliosis rather than the commoner hyperlordosis). In others, however, especially in infantile-onset FSHD with very short p13-E11 fragments, presumed primary diaphragmatic and/or intercostal muscle involvement may very rarely cause respiratory failure (Nakagawa *et al.*, 1996, 1997).

The family presented by Bailey *et al.* (1986) is noteworthy because of the death of four males under the age of 20 years (two at 15 and 16 years of age). Clinically, the cases examined fit an FSHD phenotype very closely and appeared to be inherited in autosomal dominant manner. Two cases had a sufficiently severe course to have previously been labelled as Duchenne muscular dystrophy, but there is at least one instance of male-to-male transmission. Atypical features include ptosis, bulbar weakness and calf pseudohypertrophy, each in at least two members of the family, and weak neck extensors in the proband. Two males are said to have had respiratory insufficiency and the proband, who also had pectus excavatum and bulbar weakness, suffered with repeated respiratory infections. There are enough atypical features to cast some doubt upon the diagnosis in this family. However, the clinical overlap is striking and without further specific molecular or biopsy evidence it would be difficult to discount this family entirely.

Other atypical cases with early childhood death or respiratory failure are described by McGarry et al. (1983), Yasukohchi *et al.* (1988), Saito *et al.* (2000) and Shigeto *et al.* (2002). The last article come from the Japanese literature—no molecular details are given in the English abstract. To the author's knowledge, no cases of even severe infantile or

childhood FSHD have been described with respiratory failure and a short p13E-11 fragment. Some cases of FSHD are undoubtedly sufficiently severe that they will follow a similar course to that of DMD. However, many of the previously reported literature cases are likely to represent other diagnoses, or FSHD in combination with other diagnosis (e.g. Sakuma et al., 2001). Respiratory failure probably does occasionally occur in FSHD, and may even be under-reported, but current evidence suggests it is an extremely rare complication, usually confined to the most extreme and most severely affected cases.

2.2.2 Age of onset

As with many slowly progressive neuromuscular disorders, the onset of symptoms in FSHD is usually better appreciated retrospectively once a diagnosis has been reached rather than noted at the true onset of difficulties. There are two common ways in which the diagnosis is first suspected. Firstly, in early childhood, parents may note the paucity of facial expression or the facial similarity with affected members, and realize their child is affected many years before the onset of other symptoms. Secondly, the affected individual may notice shoulder girdle problems—such as difficulty raising or keeping the arms above shoulder height, or because they have been teased about unsightly 'chicken-wings' scapulae. Since these signs or symptoms are very subjective, it is difficult to identify a precise date of onset. The given age of onset will vary enormously, therefore, depending on whether the onset of facial signs or of shoulder weakness is accepted. This can be a particular problem when looking at issues such as anticipation. Most authors take the onset of symptomatic weakness, usually in the shoulder girdle, to be the more comparable and clinically significant figure.

More than 95% of cases of FSHD will follow the classical adult-onset course. In his group of 73 patients, Padberg (1982) found a mean age of onset of 17.8 years. Lunt and Harper (1991) examined 11 families in which at least six individuals were affected. They noted the typical age of onset to be in the mid- to late-teens with a median age of 18 years, 95% of patients showing signs before the age of 20 years, and 99% before 30 years. Sporadic cases are known to have a much earlier age of onset, and so were excluded. Jardine *et al.* (1994b) looked at this issue in 27 new mutation cases noting a mean age of onset of 6.8 years. In one family with a scapulohumeral distribution of weakness and no facial signs, the median age of onset was 20 years (Jardine *et al.*, 1994a). Other authors have distinguished male and female patients, noting that 95% of male patients develop signs before 30 years whilst a significant proportion of women develop features later than 30 years (Padberg, 1982; Zatz *et al.,* 1998). This is the classic age of onset, but in a small proportion of cases, fewer than 5%, patients develop an early-onset and at times severe infantile form of FSHD (Padberg, 1982; Brooke, 1986; Lunt *et al.,* 1989b; Lunt and Harper, 1991; Brouwer *et al.,* 1994, 1995). This form of FSHD can be so severe that presentation may occur very early in infancy with sucking and feeding difficulty, and marked facial weakness, including in rare cases weakness of the tongue and palate (Korf *et al.,* 1985; Yamanaka *et al.,* 2001, 2002). Even in these cases, true limb weakness does not occur for a few years, but once the proximal weakness develops the course is rapid and may take a course even more severe than Duchenne or SCARMD (severe childhood autosomal recessive muscular dystrophy—now known to be one of the

sarcoglycanopathies) (McGarry *et al.*, 1983; Bailey *et al.*, 1986, Nakagawa *et al.*, 1996, 1997). At the other extreme, mild cases of FSHD have been reported right up to the sixth decade (Padberg, 1982; Lunt and Harper, 1991; Lunt *et al.*, 1995). Although the chance of this happening beyond 30 years is low, it is therefore not possible to exclude completely inheritance of FSHD on clinical grounds alone.

In summary, therefore, the median age of onset of FSHD is around 17 years, but with a wide range, varying at the most extreme from infancy to the seventh decade. New mutation cases have an earlier mean age of onset, whereas other selected groups and females may have a later age of onset.

2.2.3 Penetrance

FSHD is a true autosomal dominant muscular dystrophy. In their original papers, Landouzy and Déjérine noted: 'hereditability is clearly demonstrated by the presence of a progressive muscular atrophy in the father, brother and sister'. In Tyler and Stephens' study of a large Utah family (1950), 130 offspring were affected and 143 were normal. This gave a ratio of 1.0:1.1 leading Tyler and Stephens to conclude that FSHD showed 'a typical Mendelian dominant pattern of inheritance with complete penetrance and variable expressivity'. After excluding probands, Becker (1953) noted that 41% of siblings were affected and 46% of the affected parent's siblings were also affected. Kazakov *et al.* (1974) studied their own and 145 literature cases. Taking the offspring of an affected parent, they found 49% to be affected. Both these groups also concluded that penetrance was complete. Padberg (1982), after exclusion of probands to remove ascertainment bias, examined 165 sibs, noting 80 to be affected and 85 unaffected. Two individuals had minimal signs and were classified as *abortive* cases, suggesting to Padberg that penetrance need not be complete. Using regression curves, Padberg calculated that 2.5% of gene carriers would not have expressed the disorder by 57 years of age, although 94.3% were clinically affected by 20 years. In 1989, Lunt *et al.* estimated penetrance after excluding probands as well. They derived age-related penetrance figures of <5% for ages 0–4 years, 21% by 10 years, 58% by 15 years, 86% by 20 years and 95% above 20 years. Their actual figures suggested 100% penetrance by the age of 20, but in a wider gene mapping study, two obligate carriers were clinically unaffected at 42 and 51 respectively, leading him to lower the upper limit to 95%. Based on sibling studies, most European authors have considered penetrance to be probably close to 100% by 30 years. A study of 53 Brazilian FSHD families by Zatz *et al.* (1998) suggested a significant difference between males and females. They found an age-related penetrance of 95% for males by 30 years, but only 69% for females, giving a combined penetrance of only 83% by 30 years (see below for further comments). Even with elucidation of part of the molecular answer, overall estimates of non-penetrance may still be as high as 5%, but until the exact underlying molecular cause of FSHD is identified, true penetrance figures will not be known. The situation is further complicated by identification of mosaic cases, who are likely to account for a significant proportion of *abortive* cases.

2.2.4 Gender differences

Early studies did not distinguish between age of onset, severity or gender-related penetrance. Becker (1953) first raised the issue of difference in severity of affected female patients and Chung and Morton (1959) a gender difference in the age of onset. Of probands examined by Padberg (1982), 15 of 19 were male. Excluding facial symptoms, the overall mean age of onset was 17.8 years, but this obscured a mean age of onset of 16.1 years for males and 20.6 for females. Considering abortive cases at all ages, 22% of males and 44% of females were asymptomatic but this difference was not statistically significant. In the previously mentioned study by Zatz *et al.* (1998), penetrance figures for males were 95% by 30 years and 69% for females by the same age. Their data, however, are heavily, and maybe correctly, skewed by six of 27 families where inheritance at first appears to be X-linked or autosomal recessive. They produced few supportive clinical details; three families only amount to one sibship and two of the other three, although involving three generations, exhibited no instance of male-to-male transmission. Molecular studies involved single-digest *Eco*RI only, with double digest confirmation of one affected member only. One of the single sibship parents exhibited molecular mosaicism, and so should be excluded from analysis. In a second sibship, neither parent was available for study, either to confirm their unaffected status or to exclude mosaicism. A further family was referred with a probable diagnosis of Becker muscular dystrophy. The maternal grandfather and four maternal greatuncles were all affected (no clinical details given). The proband carried a 25 kb *Eco*RI fragment. The mother and a brother who were asymptomatic both carried the same short fragment. From the details given this family still looks convincingly X-linked. The issue of translocation between 4q35 and 10q26, which may occur in up to 20% of the normal population, as a potential explanation is not addressed (van Deutekom *et al.*, 1996). Amongst other rarer possibilities, this could be a Becker family with a coincidental *Bln*I resistant short fragment. This argument is less plausible for a fourth family where co-segregation of a short fragment is demonstrated in four affected males and an obligate carrier mother/sister, but male-to-male transmission has not occurred; we do not know if other unaffected members of the family were tested. There are in essence many questions about this particular paper and yet there is much evidence suggesting that there is a gender-related difference in penetrance, age of onset and clinical severity. As Padberg indicates, ascertainment and reporting bias may falsely suggest a gender effect. In his own study, there was no statistically significant difference in numbers of affected members of each sex (Padberg *et al.*, 1995b). However, there was a significant difference in the sex distribution of probands, which might be because males are more severely affected at a younger age, or may reflect ascertainment bias. There may be explanations such as an X-linked modifying gene, or alternatively a Y-linked gene or epigenetic effect—the Y-chromosome is known to carry a 9 kb fragment which cross-hybridizes with the probe p13-E11. It has also been noted by Zatz *et al.* (1998) that the proportion of female mosaics is significantly greater than male mosaics. Severely affected males are also more likely to have inherited a *de novo* short fragment or a maternally transmitted fragment but this is likely to be influenced by genetic fitness if males carrying the same size fragment do indeed have an earlier age of onset and more severe disease.

2.2.5 Hearing impairment

The association between hearing impairment and FSHD was first described by Small in 1968. Hearing impairment could be profound and in some families could be the presenting feature of FSHD (Taylor *et al.*, 1982). In infantile FSHD, speech and communication difficulties, caused by severe facial weakness, may be further compromised by significant hearing impairment. The major psychological, communication and learning problems consequent to this, and the delay in diagnosis, are well described by Meyerson and Lewis (1984). Most hearing problems were initially described in infantile cases, many of whom were also sporadic cases. Gurwin *et al.* (1985) described an autosomal dominant family with four affected members who also had hearing impairment. Voit *et al.* (1986) traced the source of high-frequency sensorineural deafness in infantile FSHD to the cochlea. In two cases, hearing impairment was clearly progressive. Severity was correlated with severity of FSHD but was not related to the duration of myopathic symptoms. A raised stapedial reflex threshold was also noted, but because this is also raised in other myopathic disorders, and in FSHD patients without hearing disability, they concluded that it did not play a primary role in the cause of the hearing defect, suggesting instead primary involvement of the outer hair cells.

Hearing impairment in infantile cases was also associated with other unusual features such as mental retardation and peripheral vascular retinopathy (Small, 1968; Matsuzaka *et al.*, 1986). These features and the fact that many infantile cases are sporadic new mutations led to discussion about possible genetic heterogeneity. Addressing this question, Brouwer and Padberg undertook several studies involving patients of all ages but predominantly typical adult-onset FSHD (Brouwer *et al.*, 1991; Padberg *et al.*, 1995a). In an initial study of 56 patients at home, they found a ‘significant difference in hearing thresholds’ between affected and unaffected members of the families studied. In a second audiometric follow-up study, they noted that 62.5% of familial and 71% of sporadic cases had significant hearing impairment, although the level of impairment was not defined. There was also wide intrafamilial variability in both severity of FSHD and severity of hearing impairment. In the group as a whole, there was no correlation between hearing impairment and severity of FSHD. Jardine *et al.* (1994b) found symptomatic hearing loss in 30% (8/27) of early-onset cases and asymptomatic impairment in two others. Verhagen *et al.* (1995) noted hearing impairment appeared to show a familial association. Padberg and Brouwer, in two further publications, reviewed early-onset FSHD and added ten further cases (Brouwer *et al.*, 1994, 1995). More than 50 cases had been reported, 63% of which had hearing impairment. They acknowledged that infantile FSHD only accounts for 5% of FSHD cases and that selective reporting and ascertainment bias will almost certainly affect the publication of cases. Although many cases reported in the literature were undoubtedly familial, all of their own patients with hearing impairment were sporadic cases. A recent study by Rogers and Zhao, on 21 classic adult-onset FSHD cases demonstrated no difference in pure tone hearing thresholds or prevalence of hearing impairment (Rogers *et al.*, 2002). In fact, statistical analysis suggested better hearing when compared to age- and gender-matched controls. Significant sensorineural hearing loss, therefore, appears to be a feature of early-onset and sporadic cases of FSHD, and although it may be seen in some cases, does not appear to be consistent feature of adult-onset FSHD.

2.2.6 Coats' disease and vascular retinopathy

A vascular retinopathy is undoubtedly a feature of some cases of FSHD. How common, how severe, and whether ophthalmological surveillance should be instigated are, however, more debatable. Coats' disease, a severe exudative vascular retinopathy has been described by a number of authors in association with FSHD. According to Manschot and de Bruijn (1967), Coats' re-defined this disease in his second German paper (which is often overlooked in favour of the first which was in English) as a condition which is usually unilateral, non-hereditary, with an exudative vascular retinopathy, usually affecting juvenile or infant males *in the absence of systemic disease* (Coats, 1912). (This last point being omitted by many ophthalmologists and neurologists alike.) It is often associated with massive retinal exudates, consequential retinal detachment, and blindness (Manschot and de Bruijn, 1967; Gurwin *et al.,* 1985). The first description of an association with FSHD is generally accepted to be that of Small (together with the first description of hearing impairment) in 1968. The clinical cases presented by Small as 'muscular dystrophy' are certainly convincing as cases of severe childhood-onset FSHD. The four individuals also manifest wide variability of expression of both muscular dystrophy and retinal disease from total retinal detachment or advanced 'Coats' disease' in four eyes (one had been enucleated) to telangiectasis or vascular tortuosity in the four other eyes. This suggested to Small that telangiectasis or tortuosity should be regarded as a milder form (or even precursor) of Coats' disease. For the next 25–30 years, over 55 cases of severe infantile FSHD were described, many with retinal vascular disease (reviewed by Brouwer *et al.,* 1994, 1995). The two cases described by Taylor *et al.* (1982) undoubtedly have early-onset muscular dystrophy, but importantly visual symptoms antedated weakness in both cases. In the first case, visual acuity was first noted to be decreased at age 5 years. A marked exudative retinopathy progressed to 'Coats' disease' requiring photocoagulation treatment. Muscle weakness developed at 7 years of age. Case two was found to have bilateral leucokoria and retinal detachment at 6 months of age—initially thought to be due to bilateral retinoblastoma, the correct diagnosis was only made after histological examination of the enucleated left eye. In the same journal, Wulff *et al.* (1982) reported on a 26-year-old man who was initially noted to have sensorineural deafness at 2 years of age. Weakness of facial muscles was noted at 5 years. At 13 he presented with an exudative retinopathy necessitating repeated photocoagulation yet muscle symptoms remained mild. Gieron *et al.* also described a mother and three children affected with FSHD, all of whom also exhibited marked tortuosity of retinal vessels (Gieron *et al.,* 1985). Like Small, they recognized that tortuosity of retinal vessels may be seen in up to 15% of the population. However, examining 25 control subjects they only found minimal tortuosity in one normal person. They therefore postulated that retinal vascular tortuosity may represent a mild manifestation of Coats' disease. But like other authors (Matsuzaka *et al.,* 1986), they also questioned whether the rare constellation of FSHD, hearing loss and Coats' disease represented genetic pleiotropy of FSHD or a 'new' nosological entity. Gurwin *et al.* (1985) describe a much more typical FSHD family with a clear autosomal dominant family history. One individual had a history of poor (counting fingers) vision since early childhood. Ophthalmoscopy revealed a macular loss of vision associated with a macular scar, retinal telangiectasis and foveal hyperfluorescence. Other family members, who had no visual symptoms, were all found to have varying degrees of retinal vascular leakage

on fluoroscopy, but not the 'massive retinal exudate and associated detachment' of Coats' disease. Two years later Fitzsimons *et al.* (1987), a co-author of Gurwin, reported abnormalities, including microaneurysms, retinal telangiectasis, vascular tortuosity, leakage and capillary occlusion in 75% of patients with classic-onset FSHD indicating that genetic heterogeneity was unlikely. Out of the 64 cases examined, fundoscopically visible changes were only seen in three cases and visual loss only affected one person. However, eight of 30 (27%) unaffected first-degree relatives also exhibited features suggestive of a retinal vasculopathy. In this period, prior to the discovery of the 4q35 shortened molecular fragment, or the delineation of many other neuromuscular conditions, diagnosis depended very much on the clinical description of each case. Some are certainly questionable—such as those of a brother and sister, the offspring of consanguineous parents (Yasukohchi *et al.*, 1988). The brother presented with congestive cardiac failure and respiratory failure, aggravated by obesity, whilst weakness was only mild. Other atypical features include a severe scoliosis, early weakness of neck flexion, an ability to whistle in spite of bilateral facial wasting and weakness, an atrophic tongue, the presence of pseudohypertrophy, and raised hemidiaphragms associated with his respiratory failure. Almost all of these features may occasionally be seen in FSHD, but not usually together, or early in the course of the disease. There are a number of more likely alternative differential diagnoses and a Gomori trichrome stain of the biopsy, looking for nemaline rods, would be of particular interest. Reviewing the literature plus six of their own cases, Brouwer *et al.* (1994) identified, with fluorescein angiography, retinal abnormalities in 15/23 infantile or early-childhood cases of FSHD but the significance of the finding in the absence of sight-threatening disease was uncertain. Infantile FSHD can undoubtedly run a severe course and has been described as at least as severe as Duchenne muscular dystrophy in some cases (Bailey *et al.*, 1986). As defined by Carroll and Brooke (1979) and Padberg (1982) with onset before 2 years of age and with rapid progression—infantile cases however, fewer than 5% of FSHD cases (Brouwer *et al.*, 1994). Early-onset FSHD (onset before 5 years) has been reported in 16–17% of 182 Japanese cases in two independent studies (Nakagawa *et al.*, 1997: Funakoshi *et al.*, 1998). None of the patients of Nakagawa *et al.* exhibited frank Coats' disease but three subjects (8%), all with single-digest fragments <13 kb, had significant tortuosity of retinal arterioles as well as hearing impairment. Jardine *et al.* (1994b) reviewed 31 sporadic cases of FSHD, generally regarded to be more severely affected than the more typical adult-onset FSHD but not as severe as infantile FSHD. Although 30% of this group had severe muscle disease, all had normal visual acuities and normal direct ophthalmoscopy (fluorescein angiography was not performed).

In order to try and clarify the significance of retinal disease, Padberg *et al.* (1995a) examined 39 molecularly confirmed FSHD patients, 130 controls and eight healthy parents of four sporadic cases. Fourteen patients (47%) had mildly or moderately abnormal fluorescein angiograms. None had a severe, Coats'-like picture. Of control angiograms, 8% were mildly abnormal and 19% equivocal. Of even greater interest, all four parents of two sporadic cases had abnormal angiograms. The angiograms of the affected offspring were normal, as were the angiograms of the two parents of another sporadic case. In the remaining family, one parent had an abnormal and one a normal angiogram. There is nothing to suggest any of the parents were mosaic for FSHD. Whilst the relative high frequency of mild retinal vascular changes in FSHD is supported, the

specificity and clinical significance of the finding is questioned. As an addendum to the paper, Padberg *et al.* also noted that Coats' disease was only seen in three of 256 Dutch FSHD cases, representing 1.2% of the FSHD population. In a later paper, Fitzsimons (1999) acknowledges that in only a small percentage of FSHD cases does the vascular retinopathy threaten sight. However, because timely photocoagulation may preserve sight (Desai and Sabates 1990), although not always (Fitzsimons, 1999) Fitzsimons recommends that patients should be aware of retinal vasculopathy and the importance of reporting unexplained visual loss. Overall the literature would suggest that whereas sight-threatening vasculopathy may rarely affect older patients, most examples of severe retinal disease have occurred in childhood onset (Desai and Sabates, 1990) or infantile cases, usually in association with significant hearing impairment, and/or in cases with p13E-1 1 fragments less than 13 kb. An important caution however is the fact that even in these patients, a number of cases of severe eye disease have antedated muscle symptoms (Taylor *et al.*, 1982; Gurwin *et al.*, 1985; Fitzsimons *et al.*, 1987). It may, therefore, seem more prudent that all patients should be regularly asked about visual loss and as recommended by Fitzsimons (1999) that 'all infants at genetic risk of FSHD (and particularly those with a family history of retinal complication)', should undergo occasional indirect ophthalmoscopy. This author would also add those families with a family history of hearing loss (>25 dB at 4 or 6 kHz) or double-digest fragments less than 15 kb and certainly those less than 13 kb.

2.2.7 Cardiac involvement

Other muscular dystrophies such as myotonic dystrophy and Emery-Dreifuss muscular dystrophy (EDMD) are well known to cause cardiac complications—either due to cardiomyopathy, or to selective involvement of the conducting system (Emery, 1987b; Oswald *et al.*, 1987; Hawley *et al.*, 1991; Phillips and Harper, 1997; Lazarus *et al.*, 1999). The Xp21 dystrophies on the other hand are also associated with both dysrhythmias and cardiomyopathy, and the autosomal dominant limb girdle muscular dystrophy, LGMD1B, with cardiac conduction block (Emery, 1987b; de Visser *et al.*, 1992; van der Kooi *et al.*, 1996b, 1997; Fang *et al.*, 1997). In spite of this, and the potential seriousness of cardiac involvement, this subject has not drawn as much attention as it might. Tyler and Stephens (1950) in their large Utah FSHD family noted a high prevalence of cardiac disease. The prevalence of rheumatic fever was high in Utah anyway (seven per 1000 high school students) but the prevalence of 'signs suggestive of rheumatic heart disease' was 12 times higher at 87 per 1000 members of the family. This was noted as an observation only, so no attempt was made to confirm the presence of rheumatic heart disease or to explain whether there were other immunological, nutritional or social factors to explain this finding. No other cardiac studies have repeated this finding in other families. Other early studies were hampered by the probable inclusion of other disparate diagnoses now known to be associated with cardiac disease such as EDMD and scapuloperoneal syndrome with cardiomyopathy. Such a study is that of Manning and Cropp (1958) who compared standard 12-lead electrocardiograms of 28 cases of juvenile muscular dystrophy with 10 patients who had adult onset muscular dystrophy *or* FSHD. They found that more than 70% of the 'juvenile group' had abnormal ECGs but in spite of the inclusion of other diagnoses still found 'little or

nothing abnormal' in the FSHD group. Kilburn *et al.* (1959) also lumped FSHD and LGMD cases together.

Clinical descriptions suggest that only two of these cases fitted an FSHD phenotype. Of these, one exhibited ECG changes compatible with an incomplete right bundle bunch block (RBBB), but otherwise had no specific cardiac symptoms. Caponnetto *et al.* (1968) and Baldwin *et al.* (1973) undoubtedly demonstrated atrial standstill in their patients. However, all these patients were male and others including Stevenson *et al.* (1990), de Visser *et al.* (1992) and Laforêt *et al.* (1998) have criticised the validity of the underlying diagnosis. Stevenson *et al.* (1990) attempted to find evidence of atrial standstill in FSHD. On standard 12-lead ECG, they found minor p wave abnormalities in 60% (18/30) of patients. Twelve patients also underwent invasive intracardiac electrophysiological studies with direct stimulation of the atria inducing arrhythmias in 80% of patients compared to 17% of controls. In keeping with a general conduction disorder, three patients had mild prolongation of the PR interval, two patients exhibited right bundle branch block and one left anterior hemiblock. The authors concluded that, in patients with FSHD, the heart was unusually sensitive to inducible arrhythmias but they were unclear about the clinical significance of these findings regarding them as 'a comparatively benign form of the electrophysiological cardiac involvement (seen) in the phenotypically similar Emery-Dreifuss muscular dystrophy'. The Dutch group of de Visser *et al.* (1992) compared Becker and facioscapulohumeral muscular dystrophies with Bethlem myopathy, performing standard ECG, M-mode and two-dimensional echocardiography, and in the case of FSHD also 24-hour Holter-monitoring. Apart from one patient with mitral valve prolapse, cardiac changes were absent in the remaining 27 FSHD patients. Thallium-201 single-photon-emission computed tomography (Tl-201-SPECT), in conjunction with a pharmacologically induced (dobutamine) stress test, was used to evaluate 15 members of a family with molecularly confirmed FSHD (Faustmann *et al.*, 1996). Only the affected members of the family showed a reduced thallium-201 uptake reflecting, in the author's opinion, cardiomyogenic changes related to FSHD. Following these two studies, Laforêt *et al.* (1998) assessed 100 FSHD patients from 90 families with molecularly confirmed FSHD. Surface ECG showed no abnormality in 53% of patients. Minor abnormalities were seen in 38 patients including RBBB, sinus bradycardia, short PR interval, repetitive supraventricular extrasystoles and others. Conduction abnormalities were induced by intracardiac stimulation in two young patients aged 14 and 20 years. Two other patients aged 43 and 44 years had symptomatic cardiac disease, one with symptomatic bradycardia and the other with nocturnal atrial dysrhythmias. A fifth patient had a right ventricular cardiomyopathy and related ventricular tachycardia at 53 years. Four other patients over the age of 60 exhibited minor ECG abnormalities that could have been related to age or underlying cardiovascular disease. There was no correlation between severity of FSHD, age of onset, or size of double-digest fragment. No attempt was made to compare the frequency of the minor or more significant anomalies with the general or an age-matched population. In the absence of other cardiovascular risk factors, however, the authors concluded that 5% of their patients exhibited FSHD-related cardiac disease.

Thus the issue of cardiac involvement in FSHD appears to have come full circle. Early reports suggested that cardiac involvement was not a feature of FSHD. Later, other phenotypically similar disorders were thought to account for the few cases of presumed

FSHD exhibiting cardiac features. More recent studies suggest that although symptomatic cardiac disease is still rare in FSHD, possibly 5% of patients will show clear evidence of cardiac abnormality. Most of these appear to be rhythm disturbances, so it seems likely that the high frequency (38–60%) of minor ECG abnormalities, although not immediately of concern, may indicate a degree of usually minor cardiac involvement whose clinical significance is yet to be determined.

2.2.8 Early onset and rare features of FSHD

A number of other rare associations have been described in FSHD cases, often as isolated case reports and usually in severe infantile or sporadic cases. Such associations include the finding of severe learning problems, which has been estimated to affect fewer than 2% of Dutch FSHD patients (Padberg *et al.,* 1995b). Most early cases of severe learning problems were usually described in association with other findings, such as hearing impairment, retinal vascular disease or both (Small, 1968; Matsuzaka *et al.,* 1986; Brouwer *et al.,* 1994). The situation is complicated by some cases where failure to recognize profound hearing loss and/or visual impairment resulted in sensory deprivation and significant developmental delay which might not otherwise have been so marked (Meyerson and Lewis, 1984; Gurwin *et al.,* 1985). Epilepsy may be seen in some congenital muscular dystrophies—most notably the Fukuyama type with known cerebral white matter changes (Segawa *et al.,* 1979; Dubowitz, 1997), but has rarely also been described in FSHD (Akiyama *et al.,* 1991 (article in Japanese)). For an as yet unknown reason, many of the most severely affected infantile FSHD patients come from Japan. Many of these cases have the smallest fragments yet described—10 kb, single-digest, with only one D4Z4 repeat (Lee *et al.,* 1995a, 1995b). The study by Funakoshi *et al.* (1998) of 91 Japanese families identified 20 patients with early-onset FSHD (according to the definition used by Brouwer *et al.* (1994)—i.e. signs or symptoms of facial weakness before the age of 5 years, and signs or symptoms of shoulder girdle weakness before the age of 10). Of these twenty patients, 12 were new mutation and eight familial cases of FSHD. All 20 had short double-digest fragments. Comparing these 20 patients to the remaining cases, p13E-11 fragment size was significantly smaller ranging from 10–20 kb, mean 13.3 kb vs. a range of 13–33 kb, mean 18.5 kb. Hearing loss occurred in 50%, retinal abnormalities in 30%, epilepsy in 20%, and ‘mental retardation’ in 40%. Although this latter figure represents only eight (10%) of the overall 78 patients with proven short fragments, it is still significantly higher than the Dutch, and probably other Western populations. Furthermore, the eight patients with severe learning problems all had the shortest, 10–11 kb, fragments. Four of these patients also had symptomatic epilepsy (three with focal epilepsy, one with infantile spasms) all of which were well controlled with antiepileptic medication. Other case reports have identified structural abnormalities in some families including separate affected individuals with a central cleft lip and palate, and an inferior coloboma (Gurwin *et al.,* 1985) and an unaffected sibling with an encephalocele in the family reported by Small (1968). Brain imaging, by CT scan or MRI, demonstrated no abnormality in any of the four patients of Funakohshi *et al.* Severe learning problems and epilepsy *are* therefore features seen in patients at the most extreme end of the spectrum of facioscapulohumeral muscular dystrophy. They have so far only been reported in those with exceptionally small p13E-1 1 fragments, but nonetheless

together with hearing impairment, vascular retinopathy, and a severe clinical course should be anticipated in this exceptional, but small, group of FSHD patients.

2.3 Summary

Facioscapulohumeral muscular dystrophy is an inherited neuromuscular disorder first recognized as a distinct condition by Landouzy and Déjérine in 1874. It is inherited in an autosomal dominant manner. Whilst most affected people have affected relatives, there is a significant new mutation rate accounting for most sporadic cases. Occasional abortive or non-penetrant cases are encountered. The condition is generally limited to the skeletal muscles, which are affected in a characteristic distribution, with involvement of the face occurring first in early childhood for the majority. Proximal upper limb involvement occurs before proximal lower limb, although distal lower leg involvement occasionally presents first. The cardiac and respiratory muscles as a rule are not thought to be affected and do not produce clinical problems for the great majority of patients. FSHD is compatible with a normal lifespan in most individuals. Occasional severe infantile FSHD is seen as a sporadic condition, and rarely within known families. Both familial and sporadic FSHD are associated with deletions of a 3.3 kb tandem repeat unit seen in more than 95% of cases. Increasingly, unusual phenotypic variation or molecular results are being recognized, adding to the challenges presented by this uniquely fascinating disorder.

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3.
Facioscapulohumeral muscular dystrophy: a clinician's experience

George W.Padberg

FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

3.1 Historical notes

In 1885, Landouzy and Déjérine coined the adjective facioscapulohumeral to describe the hallmarks of the more advanced stage of the muscular dystrophy (FSHD) they had observed in several families and to contrast this disease with the only known muscle disease at that time named after Duchenne (Landouzy and Déjérine, 1885). Patients with FSHD had been described and even photographed before 1885 (Padberg, 1982). However, it is likely that the adjective FSH later precluded recognition of the disease in some families with lower limb involvement (Oransky, 1927; Kazakov *et al.*, 1975), while mild facial weakness in other families prompted the use of the term scapuloperoneal muscular dystrophy (Davidenkow, 1939). In any case, a discussion was necessary to get early foot-extensor weakness accepted as part of the natural course of FSHD (Tyler and Stephens, 1950; Padberg, 1982).

The second reason for the long nosological discussion on FSHD was the failure to recognize a distinct pattern of inheritance. Even when it was accepted that FSHD is usually inherited in an autosomal dominant fashion and only rarely as recessively, an unfruitful debate lingered on about sporadic cases and /or phenocopies. The more recent recognition of somatic and germline mosaicism and the high mutation rate has ended this discussion and has allowed us to draw the average picture of FSHD with more confidence and to do justice to unusual and variant manifestations.

3.2 Clinical picture

Several discussions of the clinical features of FSHD start off by stressing the interand even intrafamilial variability of the disease. What is meant by this is that the rate and

extent of progression of the disease is variable; how it begins and how it proceeds follows a rather uniform path with a very recognizable core pattern.

Facial weakness is rarely an early complaint and is only occasionally recognized by the patient as onset of the disease. Facial weakness can be found in more than 90% of cases on first clinical examination (Padberg, 1982). Several studies report lower percentages but clearly ignore minimal facial weakness thereby stressing the difficulties inherited in making such a clinical diagnosis (Felice *et al.*, 2000). We observed an asymmetrical involvement of facial muscles in more than 50% of cases and found this a helpful sign in the differential diagnosis of FSHD *(Figure 3.1)*.

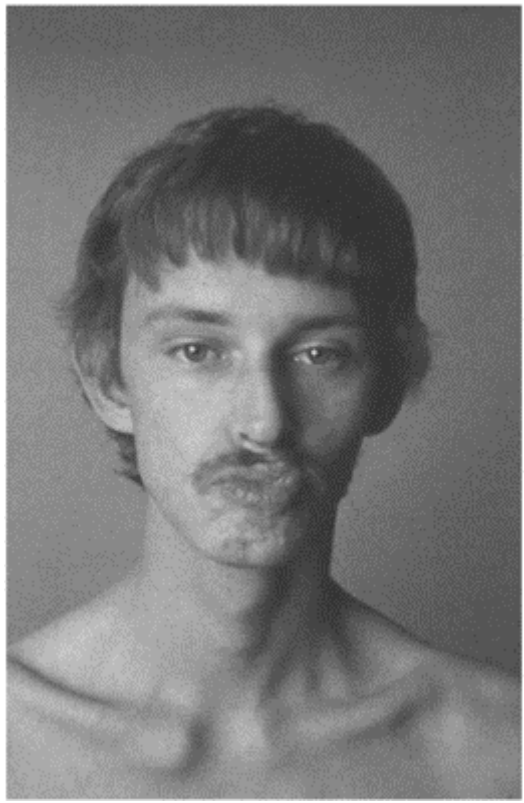

Figure 3.1 Asymmetrical facial weakness.

In our experience, more than 80% of all patients notice shoulder girdle weakness as the first symptom of the disease; approximately 5% report facial weakness, 10% foot-extensor weakness and 5% pelvic girdle weakness as the presenting complaint. The latter cases probably reflect a certain tolerance to notice or report shoulder weakness as this is present on examination in all instances. Shoulder girdle weakness usually means weakness and atrophy of the scapula fixators (m. serratus anterior, mm. rhomboideï and

the inferior part of the m. trapezius), and pectoralis muscles *(Figure 3.2)*. This weakness is often asymmetrical with unexplained preference for more involvement on the right side *(Figure 3.3)*. This preference is shared by Poland syndrome and hereditary neuralgic amyotrophy and most likely reflects a genetic mechanism. The lateral drift of the scapula over the thorax contributes to the endorotated position of the arms and the horizontal clavides at rest *(Figure 3.2)*. The shoulder girdle is also unusual in that the supra- and infraspinatus muscles

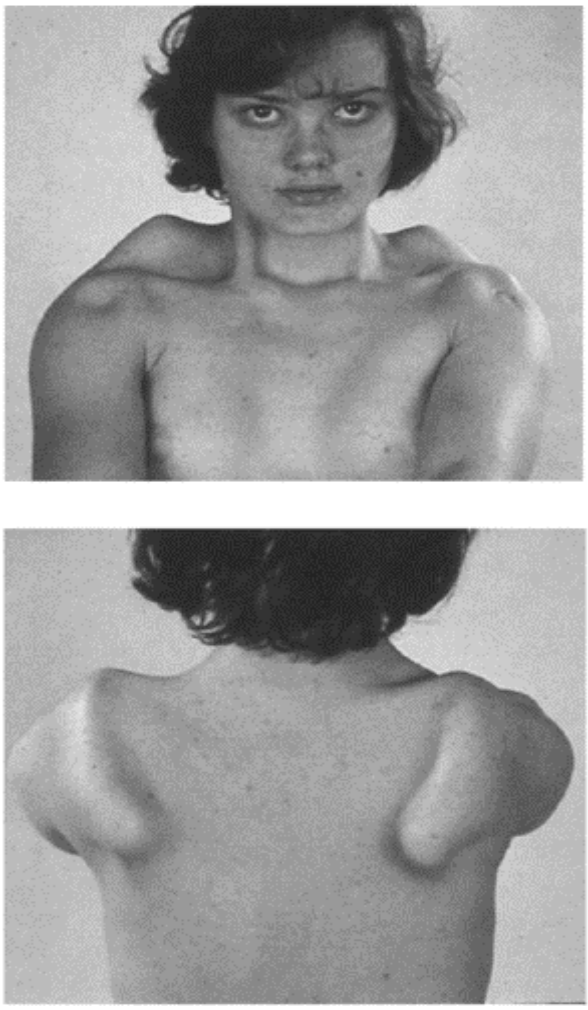

Figure 3.2 Shoulder girdle weakness with elevation of the scapulae on attempted anteflexion of the area.

frequently appear well preserved initially, whilst the deltoid muscle might even appear hypertrophic. The latter often atrophies later in the disease with marked proximal wasting *(Figure 3.4)*. The relative preservation of deltoid muscles and the severe weakness of the scapulofixators is responsible for the characteristic high rise of the winged scapula on attempted anteflexion of the arms.

A substantial number of patients—in our experience approximately 30% of all familial cases—never progress beyond shoulder weakness *(Figure 3.5)*. In the other group, the next stage in the disease involves foot-extensor weakness in 80%, or pelvic girdle weakness in 20% of patients. Some authors claim that each pattern is restricted to certain families and therefore might be determined genetically (Kazakov *et al.*, 1974). However, we have repeatedly observed both patterns within the same family.

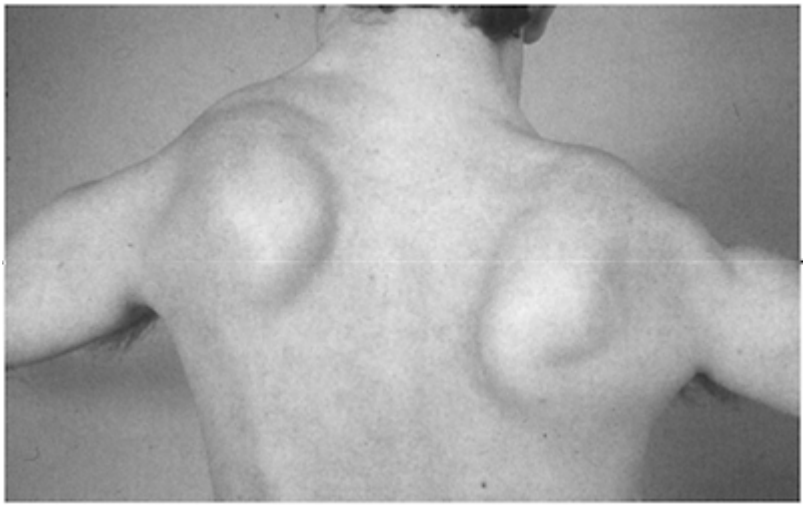

Figure 3.3 Asymmetry of muscle involvement.

At this same stage, abdominal muscle weakness is frequently found on examination; however, not all patients are aware of it until the more advanced stages of the disease. Also the biceps and particularly the triceps brachii muscles become weak and wasted around this time. Brachioradialis weakness has been noticed as a feature of this stage by some authors (Tyler and Stephens, 1950; Flanigan *et al.*, 2001) but not by others, who used the term Popeye-arms to stress the rather focal character of the myopathy with severely atrophic upper arm muscles amidst fairly normal-looking ones.

Pelvic girdle and upper leg muscles reflect the last region of involvement in most patients who progress beyond upper extremity involvement. The time to progress from foot-extensor to pelvic girdle weakness may vary extremely, from 1 to more than 25 years.

Approximately 10% of all FSHD patients and 20% of all patients older than 50 years will become wheelchair-dependent outdoors. When still walking indoors, the waddling

steppage gait with extreme hyperlordosis due to weak abdominal, gluteal and erector trunci muscles is characteristic for the disease.

In more advanced stages of the disease, the wrist extensors become weak and wrist flexors are used to aid in elbow flexion resulting in an awkward and weak grip-strength. Finger, and particularly index finger, weakness is rarely an early event and usually occurs late, if at all, in the disease. In addition, neck extensor weakness and therefore a dropped head is occasionally evident in FSHD.

It has been debated for some time whether dysphagia is part of FSHD. Although several authors agree that a number of patients, particularly with early onset of the disease, complain of swallowing problems, its true incidence is unknown. Facial muscle weakness might lead to articulation difficulties, particularly of the labials. Ligual weakness, as had been reported in some early-onset cases, will contribute to the dysarthia (Yamanaka *et al.*, 2001).

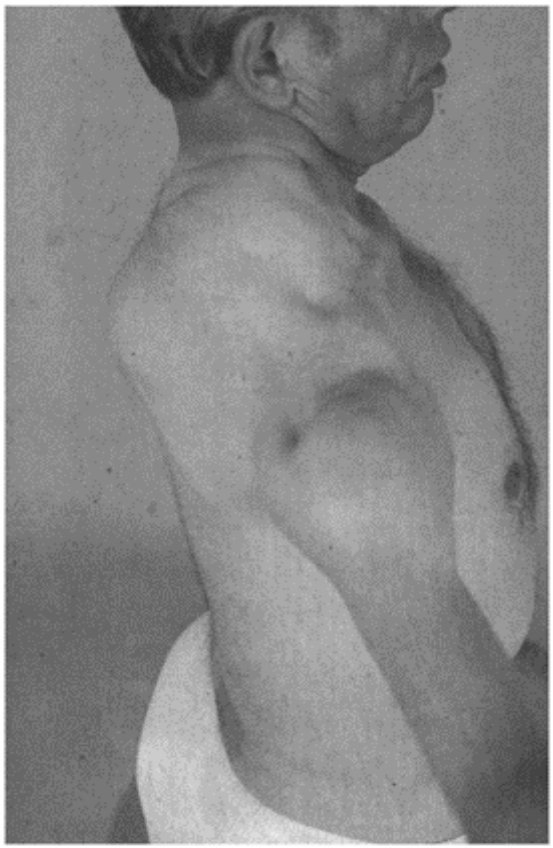

Figure 3.4 Proximal deltoid muscle wasting.

Extraocular and masticatory muscle involvement are not part of FSHD and should lead to consideration of other diagnoses. Respiratory capacity diminishes with progression of the disease but rarely leads to respiratory failure. In the Netherlands, fewer than 1% of patients are on ventilatory support. These patients are wheelchair-bound, often have severe scoliosis, and early onset of FSHD or an accompanying respiratory disease. It is suggested that such patients should be monitored when they become wheelchair-dependent.

Cardiac muscle involvement in FSHD has been a focus of debate for some time. When Emery-Dreifuss cases were recognized and eliminated from the FSHD group, only one study reported a low frequency of cardiac conduction defects in genetically proven FSHD patients (Laforet *et al.*, 1998). Other studies failed to attribute cardiac conduction abnormalities specifically to the presence of the muscular dystrophy (De Visser *et al.*, 1992).

Pain does not appear as a symptom of FSHD in the older textbooks. However, a Dutch survey confirmed a French study revealing that approximately 75% of patients relate muscle pains to FSHD. Whilst 25% of patients complained of generalized muscle pains, 25% had shoulder girdle pains related to excessive use, and

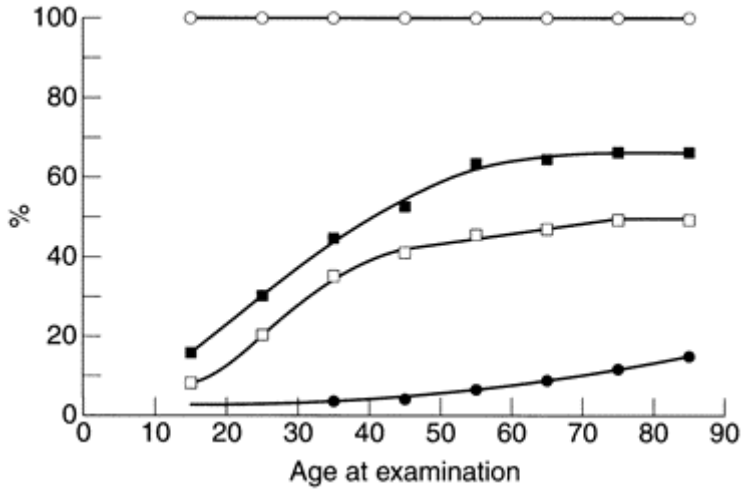

Figure 3.5 Evolution of muscle involvement, categorised per decade, in 107 examined gene carriers from 19 autosomal dominant families: ○ shoulder weakness; ■ foot extensor weakness; □ pelvic girdle weakness; • wheelchair dependency.

25% had a chronic type of shoulder pain. Treatments prescribed included non-opiate pain medication, physical therapy and reduced muscle activity; in most cases the regimen reported moderate beneficial effects.

The clinical observations mentioned above are of help in making the differential diagnosis of the FSH syndrome *(Box 3.1)*. CK-levels in FSHD are usually not more than five times the upper limit of normal and the EMG exhibits a myopathic pattern in affected muscles in most patients. Still, a high CK level or some neurogenic features in the EMG does not confirm FSHD. In our opinion, genetic testing should be the preferred laboratory test. Only when this is negative is a biopsy of an affected muscle indicated.

- Facioscapulohumeral muscular dystrophy
- Scapuloperoneal muscular dystrophy
- Limb girdle muscular dystrophy
- Proximal myotonic myopathy
- Facioscapulohumeral spinal muscular atrophy
- Scapuloperoneal spinal muscular atrophy
- Scapuloperoneal syndromes with cardiomyopathy
- Davidenkow syndrome
- Polymyositis
- Inclusion body myositis
- Acid maltase deficiency
- Mitochondrial myopathy
- Congenital myopathy

Box 3.1 FSH syndrome: differential diagnosis of FSHD.

The muscle pathology in FSHD is not specific. However, the presence of lobulated fibres, several angulated fibres and muscle infiltrates in combination with dystrophic features form a pattern suggestive of FSHD. Occasionally the infiltrates may be large, falsely suggesting myositis (Munsat, 1968; Arahata *et al.*, 1995).

3.3 Non-muscular features

Box 3.2 summarizes frequencies of extramuscular findings (Padberg, 1982; Fitzsimons *et al.*, 1987; Brouwer *et al.*, 1991; Padberg *et al.*, 1995a). A mild scoliosis can be found in approximately 30% of the cases. Only in early-onset cases might scoliosis become so severe as to compromise respiratory function. Contractures are usually mild; most frequently (10%) ankle contractures are found; elbow flexion, lower arm pronation and hip flexion contractures occurs only rarely. Reflexes are usually low to absent. Pectus cavatum is present in approximately 5% of FSHD patients, which exceeds the prevalence in the normal population to such an extent that it is likely to be a low-penetrant genetic effect (Padberg, 1982).

The subclinical retinal vasculopathy which we found by fluoresecin angiography in approximately 60% of our FSHD patients and which led to visual loss in fewer than 1% of our patients, has been discussed elsewhere (Padberg *et al.*, 1995a). Hearing loss is a

prominent feature in many patients with early-onset disease, which often necessitates the use of hearing aids. In the average FSHD patient, the hearing deficits often manifest as a high-tone hearing loss with a maximum loss at 8000 Hz, which is therefore difficult to discern from noise trauma (Brouwer *et al.*, 1991; Rogers *et al.*, 2002). Intrafamilial comparisons suggested on average a mild effect attributable to FSHD. The significance of a reported locus for hearing loss at 4q35 is at present unclear (Häfner *et al.*, 2000).

Scoliosis	32%
Ankle contractures	10%
Other contractures	5%
Pectus excavatum	5%
Subclinical hearing loss	75%
Retinal vasculopathy	60%
Visual loss	<1%

Box 3.2 Non-muscular features of FSHD.

3.4 Infantile facioscapulohumeral muscular dystrophy

Brooke (1976) introduced the term infantile FSHD to describe patients with early onset of the disease who were either sporadic cases or who had a parent with minimal or mild signs of the disease. We now know that the latter are mosaics and that the mutation rate is high in FSHD. Brouwer *et al.* (1994) demonstrated that the infantile form lies at one end of the spectrum of the disease while at the other end, lie non-penetrant gene carriers. When infantile FSHD is defined as clinical onset before the age of 10, one usually finds sporadic cases with large deletions and p13E-11 fragments with 1–3 D4Z4 repeats. These cases are occasionally diagnosed after birth as Möbius syndrome, often have normal motor milestones, and develop signs of muscle weakness after a couple of years. Although such patients have a recognizable shoulder girdle syndrome, the muscle weakness often appears to be generalized. This group is estimated to represent fewer than 5% of the FSHD population, but contributes considerably to the wheelchair-dependent group. A number of patients have been reported because of symptomatic retinal vascular disease, which was initially interpreted as Coats' disease. The high frequency of retinal telangiectasis, exudates and haemorrhages in early-onset FSHD led to the recognition of the subclinical form of retinal disease in the more common presentation of FSHD. Similarly, many infantile FSHD patients have a considerable hearing deficit although this is minimal or masked in a presbyacusis presentation in the average FSHD patient. In Japan, infantile FSHD has been observed frequently in association with mental retardation and occasionally also with epilepsy (Funakoshi *et al.*, 1998; Yamanaka *et al.*, 2001). Epilepsy has not been reported outside of Japan and might reflect a population-dependent effect of this disease comparable to the apparently reduced penetrance in females in Brazil.

3.5 Age at onset of the disease

Several authors who have examined entire families have reported that approximately 30% of gene carriers, detectable by physical signs, claim not to know they were carriers or not to have muscle weakness (Tyler and Stephens, 1950; Padberg, 1982). These percentages of asymptomatic carriers increase if probands and obligatory gene carriers are excluded *(Figure 3.6)*. It is likely, although not studied, that the number of asymptomatic carriers is influenced by the size of the deletion in the families. Our studies have suggested that the percentages of asymptomatic carriers per decade did not significantly increase after the sixth decade, suggesting a levelling off of the progression of the disease. This phenomenon appeared more prominent in females than in males. When we studied entire families, we found among all siblings at risk in the second decade of life the expected 50% of gene carriers by physical examination. However, only one-third of these individuals complained of muscle impairment. The majority of patients that become symptomatic report retrospectively the onset of the disease occurring in the second decade. Taking onset of symptoms as onset of the disease is an often-debated issue. In families that are acquainted with the disease, facial weakness is often reported as the earliest sign of the disease, but it is obviously rarely a complaint. Knowing the disease also leads to recognition of shoulder pain as an early symptom of FSHD. Against this increased patient awareness of the manifestations of the disease, stands the experience of many myologists of patients with advanced shoulder girdle weakness who did not recognize their muscle impairment as abnormal. The relativity of reported symptoms notwithstanding, we noted in our series an average age of onset of 17.0 years (SD 8.8) when observations of facial weakness were included, and 17.8 years (SD 8.1) when these were excluded (Padberg, 1982).

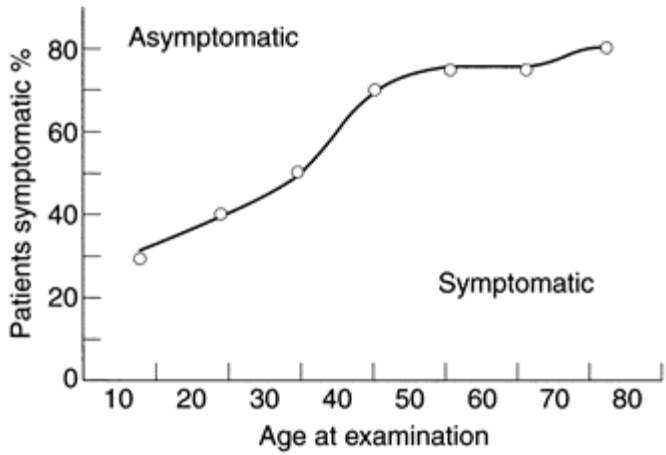

Figure 3.6 Symptoms of muscle weakness in 107 gene carriers.

3.6 Gender and generation effects in FSHD

We noted a consistent gender difference with significantly more asymptomatic females, and later onset and slower progression of the disease in symptomatic women (Padberg, 1982). This gender difference has been reported in all populations studied (Zatz *et al.*, 1998) and has been corroborated by the observation that male mosaics are more frequently symptomatic than females, and that males require considerably lower percentage of mutated cells than the female mosaics to manifest signs of the disease (Van der Maarel *et al.*, 2000). This apparent female tolerance to the disease is currently not well explained.

Anticipation, i.e. the earlier onset of the disease in successive generations, has been reported in several studies (Lunt *et al.*, 1995; Zatz *et al.*, 1995; Tawil *et al.*, 1996). Most authors relied on reported onset of symptoms; Tawil *et al.* used a normalized quantitative isometric myometry (QMT) score and arrived at similar conclusions. In our study of anticipation in 27 parent-sib pairs from which we had all clinical information, the entire (significant) effect was explained by the late age at onset in the mothers, so that we could not rule out a cultural bias. At present, no satisfactory explanation for possible anticipation is available.

3.7 Course of the disease

Normalized QMT sum-scores suggest a linear progression of the disease over time (The FSH-DY Group, 1997). A recent study, however, showed that individual muscles progress differently over time. A transverse study based on examination of all patients in 19 families *(Figure 3.5)* suggested that progression to further stages of the disease levels off after the age of 50 (Padberg, 1982). These observations strengthen occasional observations reported in the older literature of rapid progression in a short period of time and of years of apparent standstill of the disease. This has contributed to the overall impression that the course of FSHD is extremely variable. Life expectancy did not appear to be reduced in the kindreds that we studied. However, little is known about the natural course of the disease in sporadic cases which are often more severely affected.

3.8 Prevalence and fitness

In our population study in 1982, we calculated a prevalence of 1 in 46 000 individuals in The Netherlands. We also estimated that we had seen fewer than 45% of the kindreds in the population under study. Therefore, the true prevalence was estimated to be greater than 1 patient per 21 000 individuals (Padberg, 1982). By 1992 we had doubled the number of kindreds ascertained in this same population suggesting that our original estimate was close to reality (Padberg *et al.*, 1995b).

We calculated a relative fitness of 1.0 in the kindreds studied (Padberg, 1982). This, however, is not compatible with the estimated 10% of living patients being new mutations (Padberg, 1982). Although the mutation frequency in Brazil was estimated to

be even higher (i.e. 40%), a fitness of 0.6 based on familial and sporadic cases in this population appears to be more appropriate (Zatz *et al.*, 1995).

3.9 Penetrance

In our original kindreds, we observed almost complete penetrance after the age of 20 years (Padberg, 1982). These kindreds were selected through their probands for possible inclusion in genetic studies. This probably introduced a bias in favour of recognizable families with a moderate-to-severe form of the disease. In addition, since age of onset and severity of the disease roughly relates to the size of the deletion, we probably also introduced a bias toward medium-sized deletions. Limited studies of small families with small deletions suggest that the clinical syndrome might more often be mild and that penetrance might not be complete. A large number of families is needed to estimate deletion-related penetrance. A possible population effect must be addressed since gender-dependent non-penetrance based on genetic criteria appears to be fairly prevalent in Brazil (Zatz *et al.*, 1998).

3.10 Therapies

Few therapies have been worked out for FSHD. An open label study with prednisone 1.5 mg/kg/day for 12 weeks did not result in any benefit comparable to the results achieved in Duchenne patients (Tawil *et al.*, 1997). The β_2-adrenergic drug albuterol (8 mg b.i.d.) appeared to have a positive effect on muscle mass and strength in an open label trial for 3 months but proved unsuccessful in a blinded, placebo-controlled study lasting 1 year (Kissel *et al.*, 1998, 2001). A Dutch trial seeking an additive effect of training on the effects of albuterol demonstrated a small positive result of both interventions on the biceps brachii muscles but not on the foot-extensors.

Scapula fixation for FSHD has been proposed in several publications (Mummery *et al.*, 2003). The number of techniques described reinforces the impression that a standard technique still remains to be found. Scapulothoracic fusion appears to be effective but has the disadvantage of long-term immobilization that all other techniques try to avoid. On the other hand, ligatures became overstretched or led to rib fractures in a number of cases. The only technique consistently considered to have had some success is a mix of a plate and wires with a limited time of immobilization and a small effect on respiratory capacity (Berne *et al.*, 2003). The effect of scapula fixation is most pronounced when the arms cannot be raised more than 70% in anteflexion. In these circumstances, the deltoid is usually strong enough, with a fixed scapula, to abduct the upper arm to 90% and exorotate the humerus so that the flexed elbow can bring the hand easily to the level of the face and head. Such mechanisms do not allow weights to be lifted. Therefore in our opinion, such operations are of limited effect and should be recommended only with caution.

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4.
Mapping of the *FSHD* gene and the discovery of the pathognomonic deletion

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

This chapter is dedicated to the memory of Lodewijk A.Sankuijl for his inspiring contributions.

4.1 Introduction

FSHD is one of the most common Mendelian forms of muscular dystropy. The first detailed description of facioscapulohumeral muscular dystrophy was published in 1884 by Landouzy and Déjérine (1885). They reported a five-generation family (L.) with a clear autosomal dominant pattern of inheritance although the genetic terminology and knowledge was not available at that time. In a detailed study of personally examined cases, Walton and Nattras (1954) found that FSHD is transmitted usually in an autosomal dominant pattern and only rarely as an autosomal recessive trait. FSHD shows a typical pattern of muscle involvement, the first signs being weakness and atrophy of the facial and shoulder girdle muscles with subsequent progression to upper arm, foot extensor and pelvic girdle muscles. In addition to muscle involvement, retinal vasculopathy and hearing loss have been repeatedly observed (Padberg, 1982; Fitzsimons *et al.,* 1987; Brouwer *et al.,* 1991, 1995; Padberg *et al.,* 1995). Mental retardation and epilepsy have been reported in severe cases, in particular in the Japanese population (Funakoshi *et al.,* 1998; Miura *et al.,* 1998). Family studies strongly support the view that this variability in symptomatology and progression reflect pleiotropism of a single gene defect rather than genetic heterogeneity. The occurrence of isolated cases, with non-affected parents, is observed frequently (Padberg, 1982; Wijmenga *et al.,* 1992a; van der Maarel *et al.,* 2000).

4.2 Gene mapping prior to the advent of DNA polymorphisms

The intra- and interfamilial expression of FSHD is highly variable. The natural course of the disease varies considerably; 30% are more or less asymptomatic, whilst some 10% may become wheelchair bound (Padberg, 1982; Lunt *et al.,* 1989). For optimal diagnosis, genetic counselling and patient management, insight into the molecular basis of FSHD had a high priority in the 1980s. Since a specific bio-chemical defect has not yet been defined in FSHD, identification of the causative gene along 'functional genetics' lines, as applied successfully for the cloning of, for example, the globin genes, was not possible. Alternative routes to gene identification, exploiting chromosomal rearrangements such as translocations and cytogenetically detectable deletions (Francke *et al.,* 1985) were not feasible since such rearrangements have not been described in patients with FSHD. However, gene identification employing genetic strategies was emerging in the early 1980s with important developments in molecular biology and the chromosomal localization of disease genes (Botstein *et al.,* 1980). Linkage analysis was an established technique to map loci and genes to a specific chromosome. However, the number of genetic markers at that time was very limited and consisted mainly of blood groups and protein polymorphisms. With the discovery of restriction fragment length polymorphisms (RLFPs) (Kan and Dozy, 1978), and other 'random' variation (Jeffreys *et al.,* 1985; Litt and Luty, 1989; Weber and May, 1989) in the genome, perspectives changed and the implications for gene mapping were recognized.

While concepts of genetics were emerging, the first chromosomal localization studies which were initiated (Tyler and Stephens, 1950) ruled out linkage with blood groups ABO, NM and Rhesus, whilst Chung and Morton (1959) excluded P, Duffy and Kidd. In 1980, the Dutch linkage studies were initiated by collecting blood from ten Dutch multigenerational FSHD families exhibiting an autosomal dominant inheritance pattern. The families contained 69 affected and 58 non-affected sibs and 25 spouses (Padberg, 1982). All family members underwent a physical examination by the same neurologist (GWP). Owing to the age-dependent penetrance of the condition, young asymptomatic persons cannot be excluded as carriers of the FSHD gene. Our study therefore included only affected individuals, or individuals older than 20 years. At least one patient from each family had a muscle biopsy in order to establish an accurate diagnosis. All sibs underwent audiometry to look for the hearing loss associated with this condition, and in each family at least the proband was examined by fluorescein angiography of retinal vessels. The findings of this examination strongly suggested that retinal vasculopathy and hearing loss are associated conditions in these families (Brouwer *et al.,* 1991).

Initially a total of 35 different blood group markers, enzyme and protein isoforms were tested in the Dutch FSHD families. None of these markers turned out to be linked to FSHD. Weakly positive lod scores were obtained for the immunoglobulin heavy chain gene cluster (IGH) on chromosome 14q (Padberg *et al.,* 1984). Subsequent studies with the alpha-1-antitrypsin *(SERPINA1)* gene, located in the same chromosomal region, could not however confirm the mapping of FSHD to chromosome 14q (Padberg *et al.,* 1988).

4.3 Gene mapping with DNA polymorphisms

Once RFLPs became generally available for linkage analysis, these markers were used for more extensive studies in the Dutch FSHD families. Similar initiatives were launched in several other laboratories. In order to facilitate the search for the FSHD gene, an International Consortium was established in 1988 by several groups (Lunt, 1989) and this Consortium produced a first exclusion map in 1988. More than 80% of the genome was excluded as a possible site for the *FSHD* gene by using 57 markers (Sarfarazi *et al.*, 1989). The search for the *FSHD* gene proceeded rapidly and at the beginning of 1990, a total of 225 markers had been tested and almost 95% of the genome excluded. However, these exclusion data were viewed with caution owing to the lack of a complete and reliable human genetic map.

To overcome some of the problems described above, a new kind of highly polymorphic marker was applied in our effort to map the *FSHD* gene: microsatellite markers of the $(CA)_n$-type (Litt and Luty, 1989; Weber and May, 1989). The multiallelic microsatellite markers were attractive to use because of their very high information content and because they could be analysed by PCR-based technology, without the need for large amounts of DNA and probes. Their high polymorphism information content allowed us to restrict the search to a few well-characterized Dutch kindreds, since, in the case of linkage, even a single family would yield a significant lod score. In addition, even were FSHD to prove to be a genetically heterogeneous entity, the detection of linkage in a fraction of families would still be possible.

4.4 Microsatellite markers; the first linkage success

A large number of $(CA)_n$-repeat markers had recently been isolated (Weber and May, 1989) and about 80 different $(CA)_n$ repeats were available, randomly distributed across all 22 autosomes. In close collaboration with Dr J Weber (Marshfield), within 6 weeks two key families were analysed employing a total of 60 markers (i.e. more than 6000 genotypes). This amount of data could be generated by a single person, because the workload was reduced significantly by amplifying four different $(CA)_n$-repeats simultaneously (‘multiplex-PCR’). Indeed, within 6 weeks, almost 50% of the genome had been screened for the location of the gene. Importantly, one of the microsatellite markers, Mfd22, displayed linkage with the FSHD locus at a distance of 13 cM with a highly significant lod score of >6 (Wijmenga *et al.*, 1990). This FSHD localization was the first linkage success with microsatellite markers. The microsatellite marker Mfd22 (corresponding to the locus D4S171) was assigned to chromosome 4 by means of a somatic cell hybrid panel (Weber and May, 1990). These findings were rapidly confirmed and extended (Upadhyaya *et al.*, 1990; Sarfarazi *et al.*, 1992).

4.5 Probe p13E-11; the hallmark of FSHD research

A more detailed regional localization did of course require additional experiments. At this stage, we started a very fruitful collaboration with Dr Jane Hewitt and obtained the cosmid clone 13E that was isolated from a chromosome 4 cosmid library (Los Alamos) by hybridization with a degenerate oligonucleotide probe for the recognition helix 3 of the homeodomain (Burglin *et al.*, 1989). Multicolor *in situ* hybridization to interphase nuclei demonstrated that this cosmid was the most telomeric probe available, since it mapped distal to D4S139 and D4F35S1 (Wijmenga *et al.*, 1992b). After extensive subcloning efforts in both London and Leiden, an almost single-copy probe, pl3E-11, was finally isolated. On Southern blots, this probe recognizes a rather complicated polymorphic fragment pattern with different restriction enzymes. Interestingly, this probe consistently revealed a short *Eco*RI fragment, in the range of a few to approximately 40 kb, in FSHD patients *(Figure 4.1)*. Since no recombinants were seen in the entire Dutch family material, we concluded that the p13E-11 locus was very close to the FSHD locus (Wijmenga *et al.*, 1992b).

4.6 Rearrangements in *de novo* FSHD patients

Could the short 'FSHD-fragment' be indicative of a chromosomal rearrangement? Born of a work discussion one Thursday morning, it was both exciting and impressive to learn that one of us (GWP) had collected eight sporadic patients and their parents from various parts of The Netherlands over the weekend. By the following Thursday's work discussion, our PhD student, Cisca Wijmenga, had produced Southern blots of the trios, and could clearly show that most of the sporadic patients exhibited a novel fragment shorter than 30 kb, not present in either parent. We had identified the mutation in FSHD (Wijmenga *et al.*, 1992b). Soon afterwards, we identified a small family with an apparently sporadic patient, who had transmitted FSHD to one of his sons. Southern blot analysis confirmed the father to be a new mutation (mosaic), who had transmitted the rearranged fragment to his son; the founder of a new FSHD pedigree (Wijmenga *et al.*, 1992a).

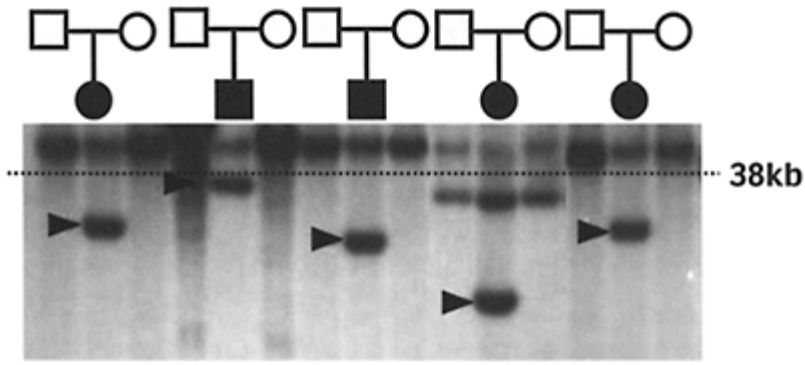

Figure 4.1 Southern blots with probe p13E-11 of *de novo* FSHD patients and their parents. In four out of five trios, a shorter fragment is seen. In subsequent generations, such a mutant fragment co-segregates with FSHD.

4.7 FSHD mutation caused by rearrangement of the 3.3 kb *Kpn*I repeat (D4Z4)

This guilt by association was a strong indication that the rearrangement had coincided with the *FSHD* gene. The next 'straightforward' step was to construct a physical map of the region and identify the gene (Wijmenga *et al.*, 1993; Wright *et al.*, 1993). Here we hit the wall that is still, some 12 years later, almost fully intact in front of us. Intriguingly, probe p13E-11 was subcloned from a cosmid clone, identified through a screen for homeodomain-containing sequences. Mapping the cosmid, we learned that it contained multiple copies of a home-odomain-containing sequence. The restriction enzyme *Kpn*I, conveniently excised each repeat unit into a 3.3 kb fragment (van Deutekom *et al.*, 1993). The official nomenclature for this repeat is D4Z4. Restriction mapping and sequencing of *Eco*RI fragments cloned from patients and controls indicated that the proximal and distal parts of the *Eco*RI fragment were identical in patients and controls. Moreover, exact sizing of the fragments in FSHD families showed that the short fragments were of different size in the different families and differed by some 3.3 kb, thereby supporting the hypothesis that the FSHD rearrangement was caused by a (recurrent) homologous recombination-based mechanism, that deleted an integral number of repeat units (van Deutekom *et al.*, 1993). Analysis by pulsed-field gel electrophoresis (PFGE), allowing separation of the long EcoRI (or *Hind*III) fragments, demonstrated that probe p13E-11 recognized two highly polymorphic loci. Haplotype analysis unambiguously assigned one of the loci to chromosome 4q35. In sporadic cases, the parental origin of the rearranged short fragment could be determined; however, no obvious gender of origin difference was detected. In a separate study, Bakker *et al.* (1995) was able to map the abovementioned

second p13E-11 locus to the tip of the long arm of chromosome 10. Since genetic heterogeneity had been demonstrated in FSHD, it was tempting to speculate that the chromosome 10 locus might harbour the FSHD2 locus. However, analysis of a large non-4q35-linked Danish family did not reveal linkage to chromosome 10q26 (Bakker *et al.*, 1995).

4.8 Does the FSHD D4Z4 array modulate long-range gene expression?

The repetitive structure of the D4Z4 locus and its telomeric localization resulted in the challenging model that the pathogenesis of FSHD is not necessarily due to the disruption of the structure of the FSHD gene, but rather to a change in its function. The basic model is that the chromatin structure determines the long-range gene expression pattern in the region (Winokur *et al.,* 1994). The length of the D4Z4 repeat, with an endogenous tendency to form heterochromatic conformations, is instrumental to the overall structure of this subtelomeric domain. Such a chromatin-based change in gene expression, similar to position effect variegation, could in principle involve a gene within each repeat unit or gene(s) in the vicinity. Although the jury is still out on this question, both options are still open. The D4Z4 repeat contains an open reading frame for the putative DUX4 sequence, promoter sequences and binding sequences for a multiprotein transcription complex (Gabriels *et al.,* 1999; Gabellini *et al.,* 2002).

Genome-wide and gene-specific expression studies have yielded some, albeit conflicting, support for overexpression of genes upstream of the D4Z4 repeat (van Deutekom *et al.,* 1996; Gabellini *et al.,* 2002; Jiang *et al.,* 2003). The relevance of these findings has to be carefully scrutinized. It should be realized that even fairly minor changes in gene expression could be sufficient to explain the relatively slow progression of the FSHD phenotype. Since FSHD in all likelihood is a congenital and systemic disorder, we do not even have an idea about the spatiotemporal requirements of correct gene expression regulation. If the envisaged position effect model proves to be valid, with multiple flanking primary genes involved, the secondary cascade of disturbed gene expression may be unpredictably large. It is even imaginable that the molecular culprit and potential intervention targets are not localized to chromosome 4.

Future experiments, including the generation of inducible and conditional animal models, have to shed more light on the FSHD molecular puzzle. It is comforting to see that an increasing number of scientists from various fields of expertise, specialized in epigenetics, embryology, DNA repair, recombination, etc., are warming up on the sidelines to join the FSHD programme; you are most welcome!

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5.
Identification and characterization of candidate genes in the FSHD region

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

5.1 Position effect as the underlying mechanism in FSHD

The nature of the DNA rearrangement associated with FSHD, a partial deletion of the D4Z4 repeat array in the subtelomere of 4q35, has seriously complicated the identification of the FSHD gene(s). Instead of a structural rearrangement (mutation) within a gene as usually found in monogenic disorders, it is becoming increasingly evident that FSHD is caused by a rather unusual genetic mechanism: position effect variegation.

The location of the D4Z4 repeat array in the subtelomeric region of chromosome 4q indicates a possible role of chromatin structure in the development of FSHD. Generally, chromatin can occur in an open and accessible structure or in a condensed state termed euchromatin and heterochromatin, respectively. Euchromatin decondenses during interphase and replicates throughout the S-phase, whereas heterochromatin is tightly packed during the cell cycle and replicates in the late S-phase (reviewed by Riggs and Pfeifer (1992)). Heterochromatin contains mainly non-coding repetitive DNA elements like satellite DNA (Hennig, 1999) and is often found in centromeric and telomeric regions. Since the FSHD locus maps adjacent to the telomere (the subtelomere) of chromosome 4q (Bengtsson *et al.*, 1994), it was hypothesized that the deletion of a critical number of D4Z4 repeat units in FSHD patients could alter the chromatin structure thereby influencing gene expression. This phenomenon, in which DNA rearrangements affect neighbouring genes, is called a position effect. Position effects have been extensively studied in *Drosophila*, where a natural chromosomal inversion placed the active *white* gene close to a heterochromatic region. The *white gene*, which is involved in red eye pigmentation, remained active in some cells, while in other cells the gene was inactivated by the close proximity of the heterochromatin. As a result, the fly showed mosaic red (active *white* gene) and white (inactive *white* gene) eye pigmentation (Muller, 1930).

Position effects can influence gene expression by several mechanisms. Chromatin rearrangements can alter the distance between an enhancer or other *cis*-acting regulatory elements and a specific gene resulting in the gene becoming more or less active. The classical type of position effect is caused by changes in chromatin condensation influencing the expression of neighbouring genes. When chromatin becomes more heterochromatic, genes can be silenced by a replication disadvantage through competition for transcription factors. In addition, the condensed structure can itself decrease the accessibility of DNA-binding proteins necessary for gene expression (Gottschling, 1992).

Position effects can influence genes proximal or distal to the rearrangements/ chromatin changes by activation or repression and can act over long (megabase) distances. This phenomenon is found in many species like *Drosophila*, yeast, plants and also in mammals (Gottschling *et al.*, 1990; Karpen, 1994; Hendrich and Willard, 1995). Chromosomal rearrangements causing position effects have also been described in human genetic diseases like thalassaemias (Townes and Behringer, 1990; Romao *et al.*, 1991), campomelic dysplasia (Wagner *et al.*, 1994; Wirth *et al.*, 1996), aniridia (Fantes *et al.*, 1995), chromosome X-linked deafness (de Kok *et al.*, 1995) and Rieger syndrome (Semina *et al.*, 1996; Flomen *et al.*, 1998).

It is proposed that FSHD is also caused by a position effect *(Figure 5.1)*. Initially, it was postulated that the D4Z4 repeat array acts as a spacer between transcriptionally active genes proximal to D4Z4 and the inactive heterochromatin distal to D4Z4 (Winokur *et al.*, 1994). Partial deletion of the D4Z4 repeat array might impair this function, causing spreading of heterochromatin and consequently silencing of otherwise active genes located proximally. In the light of recent results (Gabellini *et al.*, 2002), it is more likely that contraction of the D4Z4 repeat results in a local chromatin relaxation thereby up-regulating transcription of genes proximal (or distal) to the repeat array. Since position effects can act over megabase (Mb) intervals, the FSHD candidate region is dramatically expanded. As a consequence, a

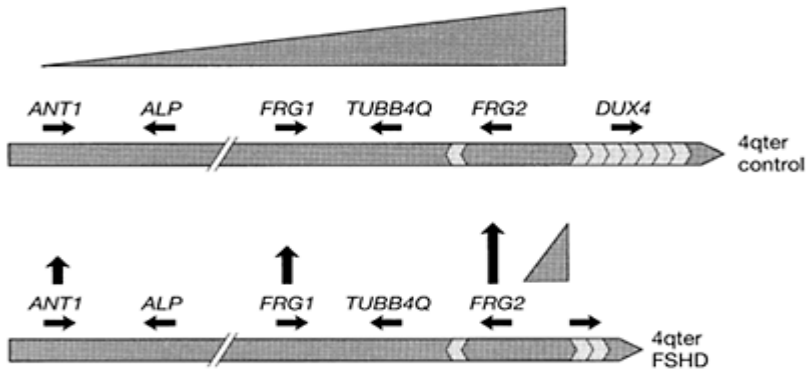

Figure 5.1 Position effect in the FSHD region. The partial deletion of the D4Z4 repeat probably results in local chromatin relaxation thereby causing

an up-regulation of genes proximal (or distal) to the repeat array. The triangles represent the influence of the D4Z4 repeat array on the local chromatin structure and therefore on the transcriptional regulation of 4qter genes. The transcriptional up-regulation of *FRG2, FRG1 and ANT1* as described in Chapter 10, is indicated by black arrows.

large region proximal to the repeat and the distal region between D4Z4 and the telomere became of interest for searching the FSHD gene(s).

5.2 The D4Z4 repeat and DUX4: the complete story?

Since the identification of the D4Z4 repeat contraction, sequence analysis initially focused on the D4Z4 repeat elements (Hewitt *et al.,* 1994; Winokur *et al.,* 1994; Lee *et al.,* 1995; Gabriels *et al.,* 1999) revealing an open reading frame (ORF) of 405 bp in each unit. This putative gene was later called *DUX4* and encodes two homeodomains. Homeodomains are involved in developmental processes and usually function as regulatory factors for transcription (Gruss and Walther, 1992; McGinnis and Krumlauf, 1992).

Human genomic phage DNA libraries, complementary DNA (cDNA) libraries and expressed sequence tag (EST) databases were screened for transcripts containing (partial) *DUX4* sequences. In great apes, homologues on different chromosomes were identified, while Old World monkeys possessed only one copy (Hewitt *et al.,* 1994; Winokur et al, 1994). Ultimately, however, none of the transcripts mapped to the 4q35 region, suggesting that *DUX4* is transcriptionally inactive (Hewitt *et al.,* 1994; Winokur *et al.,* 1994; Lee *et al.,* 1995). In a later stage, a genuine *DUX*-like transcript was identified on a non-chromosomal 4 locus, called *DUX1* (Ding *et al.,* 1998), but there is still no evidence for transcriptional activity of the *DUX4* locus.

Further complicating the potential involvement of *DUX4* is the presence of a highly homologous non-pathogenic repeat array on chromosome 10qter (Bakker *et al.,* 1995; Deidda *et al.,* 1995). Repeat arrays from chromosome 4 and 10 may recombine, resulting in translocated or hybrid alleles with chromosome 10-derived repeat sequences on chromosome 4 and *vice versa*. Nevertheless, FSHD is exclusively linked to a short D4Z4 array on chomosome 4 (van Deutekom *et al.,* 1996a; Lemmers *et al.,* 1998; van Overveld *et al.,* 2000; Lemmers *et al.,* 2001). The inability to find transcribed sequences of *DUX4*, its presence on chromosome 10 without pathogenic consequences and the clinical variation between patients with the same number of D4Z4 repeats units (see Chapter 3), indicated that an additional mechanism had to be involved in the pathogenesis of FSHD. *DUX4* is the focus of Chapter 9 and will therefore not be discussed here any further.

5.3 Gene search strategies in the FSHD candidate region

Based on the position effect hypothesis, the FSHD gene(s) should be within the immediate vicinity of the D4Z4 repeat. To define and to characterize this region and to facilitate gene identification, physical maps were required. Therefore, a long-range pulsed-field gel electrophoresis (PFGE)-based restriction map (1.8 Mb) of the FSHD candidate region was generated using rare cutting restriction enzymes (Wijmenga *et al.*, 1993). For fine mapping this region, a YAC clone (y25C2E) was used to produce a cosmid library and to construct two non-overlapping cosmid contigs, spanning 250 kb with a gap of 60 kb in the middle (Wright *et al.*, 1993). These contigs were used for subsequent gene identification approaches, like direct genomic analysis, exon trapping and cDNA selection (Altherr *et al.*, 1995; van Deutekom *et al.*, 1995, 1996b; Upadhyaya *et al.*, 1995).

5.3.1 Direct genomic analysis

Cosmids from the distal contig were subcloned and used for further gene search strategies that were based mainly on sequence information. Nowadays, the availability of the human genome sequence simplifies the identification of new genes in most regions. In the 1990s, however, when the FSHD gene search started, only a limited amount of sequence data was available. Sequencing relatively short fragments was therefore complemented by restriction and hybridization-based techniques. The subcloned cosmid fragments were used to screen for single-copy probes in the FSHD region, but only high copy number clones were found. Loci similar to D4Z4 were indeed identified on many different chromosomes, including all acrocentric chromosomes (Hewitt *et al.*, 1994; Altherr *et al.*, 1995; Lyle *et al.*, 1995).

The region telomeric to the repeat (estimated 25–60 kb) was almost unclonable. Sequence data derived from a subclone spanning 11 kb distal to the repeat reveals only highly repetitive elements and pseudogenes (van Geel *et al.*, 2002). Therefore, this region was discarded for further analysis, although recently, with the discovery of the distal variation of 4qter in which FSHD is uniquely associated with the 4qA allele (Lemmers *et al.*, 2002; van Geel *et al.*, 2002), the importance of this region is recognized. Clones mapping proximal to D4Z4 also contained pseudogenes and many repetitive sequences such as LINEs (long interspersed repetitive elements) and SINEs (short interspersed elements). The homologous areas in the genome and the highly repetitive nature of the FSHD region seriously hampered gene identification. Landmarks in the genomic region of interest, e.g. CpG islands, evolutionary conserved sequences and putative exons, helped in the search for genes.

CpG islands

CpG islands are unmethylated CG-rich regions with relatively high frequencies of CpG dinucleotides. *In silico* analysis predicts 29 000 CpG islands within the human genome (Venter *et al.*, 2001; Venter *et al.*, 2001), which are positioned at the 5′ end of approximately 60% of all human genes (Bird, 1987; Antequera and Bird, 1993). When cytosines in the CpG islands become methylated, methyl CpG binding proteins like

MeCP1 and MeCP2 can bind and thereby prevent transcription (Meehan *et al.*, 1989; Nan *et al.*, 1997; Wade, 2001). The majority of CpG islands are however unmethylated during all developmental stages in all cell types (Antequera and Bird, 1993). Unmethylated CpG islands are necessary but not sufficient for gene expression since some inactive tissue-specific genes are known to be associated with unmethylated CpG doublets (McKeon *et al.*, 1982; Bird, 1987).

Since CpG islands are frequently associated with active genes, these regions can be used as a landmark to localize new genes. When sequence data are not available, rare cutter methylation-sensitive restriction enzymes can be used to identify CpG islands, since CpG islands will be enriched for these CG-rich restriction sites. Alternatively, the combination of the frequent cutting methylation-sensitive enzyme *Hpa*II and its methylation-insensitive isoschizomer *Msp*I can be used to find CG doublets and to determine their methylation status. Finally, methylated DNA-binding columns are used to isolate CpG islands and to construct CpG libraries (Cross *et al.*, 1994).

In the search for the FSHD gene(s), the CpG island strategy proved to be successful. CpG doublets were identified within each D4Z4 repeat unit (Wright *et al.*, 1993), but also approximately 120 kb upstream of the D4Z4 repeat. This island was shown to be associated with FSHD Region Gene 1 *(FRG1),* the first candidate gene for FSHD (see Section 5.4.1).

Evolutionarily conserved sequences

Genes with an essential function are likely to have conserved orthologous sequences in other species that might provide clues for their biological role and which could be used to identify new genes. Conserved sequences can be identified by hybridization of 'Zoo-blots' with radioactively labelled fragments of the genomic region of interest. Zoo-blots are Southern blots containing genomic DNA derived from various organisms (Takiguchi *et al.*, 1993). Fragments that cross-hybridize with the genomes of other species are candidates for containing a conserved exon and these may be subsequently sequenced to search for ORFs. Interesting fragments are used as probes to identify homologous sequences in a cDNA library. Unfortunately, the subcloned cosmids from the FSHD candidate region that were used as probes to hybridize against Zoo-blots, failed to find putative exons owing to the highly repetitive nature of the region (van Deutekom *et al.*, 1995).

Gene prediction algorithms

Since almost the entire human genome is now sequenced, computer-based analysis can be very useful in the identification of a gene. Sequences of genomic DNA can be aligned with sequences from cDNA libraries and EST databases, to identify transcribed regions. *In silico* prediction programs can also be used for gene search strategies. Since exons are defined by a splice donor and splice acceptor site, several programs can indicate putative exons.

45.3.2 Exon trapping

Another approach to gene identification, based on the functional identification of splice donor and splice acceptor sites, is the search for exons *in vitro* by exon trapping (Buckler *et al.*, 1991). For this approach, genomic DNA from the region of interest is subcloned in exon-trap vectors. These vectors consist of two exons flanking an intron, in which the genomic DNA fragments are cloned. Subsequently, the library is transiently transfected in eukaryotic cells to produce large amounts of RNA, containing only exon sequences due to splicing mechanisms. RNA derived from empty vectors or from vectors which contain exclusively intronic sequences, will only consist of vector exons. However, clones containing genomic fragments harbouring one or more exons surrounded by partial intron sequences, lead to RNA molecules in which the exon derived from the genomic fragment is inserted between the splice donor and acceptor sites of the two vector exons. The major drawback of exon trapping is the need for exons flanked by intronic sequences. As a consequence, intronless and single intron genes will be missed by this technique. In addition, due to the subcloning of small fragments, clones with only exonic sequences can occur. However, the probability of such clones is low, since cells show large introns and small exons.

Exon trapping was very successful in searching genes in the FSHD candidate region (van Deutekom *et al.*, 1996b). A total of 472 exon trap clones was generated from the 150 kb distal cosmid contig. Approximately 10% of the clones were cryptically spliced or contained repetitive sequences. Analysis of all the clones resulted in the identification of five exons belonging to the FSHD candidate gene *FRG1* (see Section 5.4.1) and one cryptically spliced exon belonging to a new member of the tubulin gene family, named *TUBB4Q* (see Section 5.4.2).

5.3.3 cDNA selection

cDNA libraries can be screened for transcribed sequences using probes generated from a genomic region of interest. As this technique is very laborious for large regions, direct cDNA selection is a good alternative. For this approach, genomic DNA is immobilized on a membrane, blocked for repetitive sequences and hybridized with complete cDNA libraries. After stringent washing procedures, only specific cDNA clones are eluted from the filter. These clones can be amplified by polymerase chain reaction (PCR) and sequenced. Direct cDNA selection is less suitable for regions containing highly repetitive sequences or homologous loci in the genome, since the majority of the eluted clones will map to other regions. As the FSHD region is notoriously rich in repetitive sequences, no functional 4q35 transcripts were identified using this strategy (van Deutekom *et al.*, 1995).

5.3.4 Direct sequence analysis

The repetitive nature of the candidate region on 4q35 hampered the search for the FSHD gene(s). To bypass homology and repetitive sequence problems, PAC clones derived from this region were subjected to random sequencing (van Geel *et al.*, 1999). The direct

sequence approach confirmed the presence of many pseudogenes and repetitive sequences on 4q35 and proved that the FSHD region was gene-poor. However, the sequence data combined with computer-based gene prediction analysis allowed the identification of the FSHD Region Gene 2 *(FRG2)* and the 'reidentification' of *FRG1* and *TUBB4Q*.

This random-sequencing project had already been initiated in the early days of the Human Genome Project. The sequence analysis strategy has nowadays become less useful with the completion of the sequence of the human genome. However, small gaps still need to be sequenced, especially in the more repetitive regions such as subtelomeres. The genomic analysis of the FSHD region and its relevance for the disease are described in Chapter 8.

5.4 The most promising candidate genes for FSHD

All the different gene identification strategies described above initially revealed four candidate genes (apart from *DUX4*) in the gene-poor FSHD region, namely *FRG1*, *TUBB4Q*, *ANT1* and *FRG2* (Figure 5.2).

5.4.1 FRG1, a highly conserved nuclear protein

The identification of FRG1 by CpG island and exon trap strategies

Analysis of the cosmid contigs from the FSHD candidate region resulted in the identification of a putative CpG island in an *Eco*RI fragment of cosmid CT171, approximately 120 kb proximal to the D4Z4 repeat (Wijmenga *et al.*, 1993; Wright

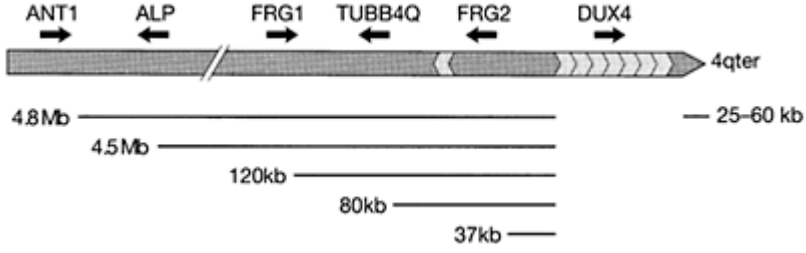

Figure 5.2 The position of the candidate genes studied in the FSHD region on 4q35.

et al., 1993). A *Pst*I subclone (p10B) of this region was used as a probe and identified a cDNA clone (10B7) from a human skeletal muscle library. The 10B7 clone was subsequently hybridized to total RNA derived from lymphocytes, fetal brain and placenta and showed a 1.1 kb fragment on Northern blot. Different cDNA libraries were used to amplify additional fragments of the novel transcript and a 5′ RACE (rapid amplification

of cDNA ends) was performed to obtain the transcriptional start site. Complementary exon trapping resulted in the identification of five exons, that appeared to be part of the same novel transcript, termed FSHD Region Gene 1 (*FRG1*, GenBank Accession number L76159) (van Deutekom *et al.*, 1996b).

The *FRG1* transcript is 1042 basepairs in length and is distributed over nine exons encoding an ORF of 258 amino acid residues. Moreover, the transcript, which lacks TATA box but possess a poly-A tail, is ubiquitously expressed.

FRG1 is a member of a multigene family

The number of *FRG1* copies in the human genome was determined by FISH (fluorescent *in situ* hybridization) experiments, in which *FRGI*-exon-containing fragments were used as probes on metaphase spreads. Complementary FRGI-specific PCRs were performed on a monochromosomal somatic cell hybrid mapping panel (UK HGMP Resource Centre) (van Deutekom *et al.*, 1996b). Multiple *FRG1*-related sequences were present in the genome, including the pericentromeric region of chromosome 9, the short arm of all acrocentric chromosomes (13, 14, 15, 21 and 22), the centromeric region of chromosome 20 and the chromosomes 8, 9 and 12. Interestingly, many homologues were mapped to heterochromatic regions in the genome, in accordance with the localization of the *FRG1* gene on 4q35. Most *FRG1* homologues exhibited in-frame stop codons and splicing aberrations such as missing exons and intronic sequences within the cDNA (Grewal *et al.*, 1999). As a result, *FRG1*-related sequences seem to be non-processed pseudogenes, some of which are transcribed (Grewal *et al.*, 1999).

FRG1P, a highly conserved protein

The *FRG1* protein (FRG1P) is highly conserved between vertebrates and non-vertebrates; the human protein is 97% identical to murine Frglp and 72% and 45% identical to its orthologues in *Fugu rubripes* (pufferfish) and *Caenorhabditis elegans* (nematode), respectively. Database searches also identified potential orthologues in, e.g. *Drosophila melanogaster, Xenopus laevis, Danio rerio* (zebrafish), tomato and *Schizosaccharomyces pombe (Figure 5.3).*

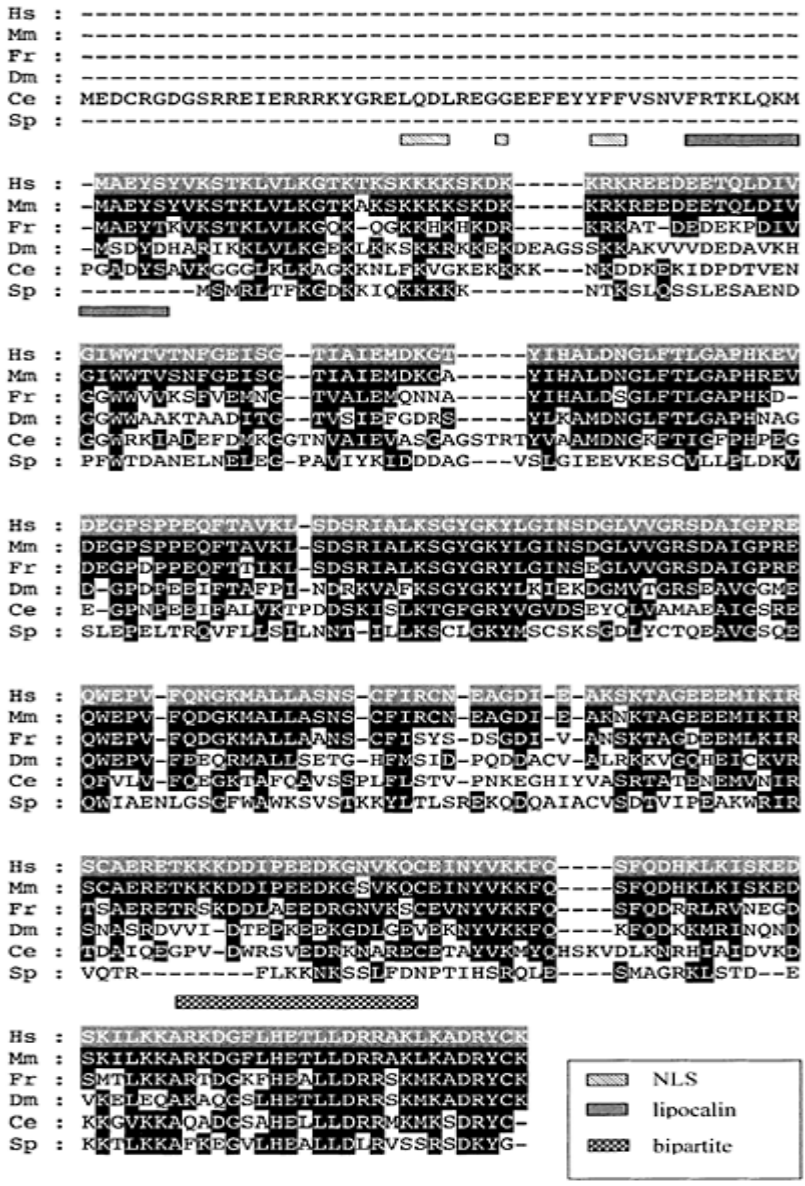

Figure 5.3 The high evolutionary conservation of FRG1 based on the human sequence. The putative nuclear localization signals (NLS and bipartite) and the predicted lipocalin motif, based on the human sequence, are

indicated with bars above the aligment. Hs: *Homo sapiens,* Mm: *Mus musculus,* Fr: *Fugu rubripes, Dm: Drosophila melanogaster,* Ce: *Caenorhabditis elegans,* Sp: *Schizosaccharomyces pombe.*

Since homologous regions in other species can provide clues for the function of a gene, physical and transcription maps of mouse and pufferfish were generated (Grewal *et al.,* 1998). Pufferfish is frequently used in gene identification approaches because it shows a reduced complexity of non-coding regions. As a result, the pufferfish orthologue of *FRG1* only spans 4.3 kb genomic DNA, a five-fold reduction as compared to the 22.5 kb human and mouse genes (van Deutekom *et al.,* 1996b; Grewal *et al.,* 1998; van Geel *et al.,* 1999). The transcript contains all nine exons with conserved positions of the intron boundaries and a poly-A tail, but with smaller intron sequences (Grewal *et al.,* 1997).

Amplification and dispersion of FRG1 during evolution

In accordance to the human genome, great apes (chimpanzee, gorilla and orangutang) show the presence of multiple *FRG1*-related sequences in their genome. However, mice contain only one *Frg1* copy (Grewal *et al.,* 1997), whilst the genome of the Old World monkey *Macaca mulatta* (MMU) exhibits two loci of which one displays two missense mutations at evolutionarily highly conserved positions (Grewal *et al.,* 1999). These results indicate a duplication of *FRG1* during the evolution of the Old World monkeys, followed by further dispersal in the great apes to multiple copies in the heterochromatic regions.

Transcriptional upregulation of FRG1

First attempts to detect aberrant *FRG1* mRNA expression levels between patients and controls using an allele-specific single-strand conformational polymorphism (SSCP) analysis failed using RNA from muscle and lymphocytes (van Deutekom *et al.,* 1996b). However, it was recently demonstrated that *FRG1* is transcriptionally up-regulated in FSHD muscle biopsies by Southern blotting (Gabellini *et al.,* 2002) and real-time PCR (our unpublished results). Interestingly, the *FRG1* expression in peripheral blood lymphocytes is not altered in FSHD patients (Gabellini *et al.,* 2002).

Functional analysis of the FRG1 protein

FRG1P shows no homology to any known protein. Detailed computer analysis predicts a putative lipocalin structure (Grewal *et al.,* 1998) *(Figure 5.3)*. Members of the lipocalin gene family mostly have three conserved lipocalin motifs and are involved in the transport of small hydrophobic molecules (Flower, 1996). The functionality of the lipocalin motif in FRG1P is questionable because it consists of only one of the three conserved motifs and the structure seems not to be conserved in all FRG1P orthologues.

Furthermore, FRG1P shows two putative nuclear localization signals: a N-terminal NLS and a bipartite sequence at the C-terminus *(Figure 5.3)*. NLS signals consist of four basic amino acid residues, whereas a bipartite sequence is composed of two basic domains separated by ten amino acid residues (Dang and Lee, 1989; Dingwall and Laskey, 1991; Robbins *et al.*, 1991).

To obtain more insight into the biological function of FRG1P, the subcellular localization was studied after transient and stable transfections in several cell lines (van Koningsbruggen *et al.*, in press). Co-localization studies with fibrillarin and coilin demonstrated that FRG1P is localized in nucleoli and Cajal bodies. The nucleolus is a membraneless structure that is organized in three components: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC). Nucleoli are mainly involved in rRNA ribogenesis, maturation of small nuclear ribonuclear proteins (snRNPs) and ageing (Melese and Xue, 1995; Johnson *et al.*, 1998; Pederson, 1998; Sleeman *et al.*, 1998; Scheer and Hock, 1999). Cajal bodies are small nuclear structures that vary in number between one and ten per cell. These bodies are thought to be involved in the post-trancriptional modification of small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) and in shuttling snRNPs from the nucleoplasm to the nucleus (Bohmann *et al.*, 1995a, 1995b; Sleeman *et al.*, 1998; Narayanan *et al.*, 1999; Darzacq *et al.*, 2002; Verheggen *et al.*, 2002). Since both structures are involved in RNA processing, FRG1P might be involved in transcriptional regulation.

The functionality of the predicted nuclear signals in transporting FRG1P to the nucleus or nucleolus was studied by subsequent deletion of these signals (van Koningsbruggen *et al.*, in press). The N-terminal NLS sequence was proven to be sufficient for the nuclear localization of FRG1P, while both signals were required for the nucleolar localization.

FRG1, the FHSD gene?

The muscle-specific up-regulation of *FRG1* in FSHD patients and its position close to the D4Z4 repeat (120 kb), make *FRG1* an attractive candidate gene. Since *FRG1* is not present on chromosome 10, which contains a repeat highly similar to D4Z4 that is non-pathogenic, this gene might explain the chromosome 4-specificity of the disease. Interestingly, several human diseases that are suspected to be caused by position effects, involve highly conserved proteins with a role in transcriptional regulation. It is hypothesized that a strict transcriptional stoichiometry is required for these proteins (Engelkamp and van Heyningen, 1996). In this respect, a potential role in RNA biogenesis of the highly conserved FRG1P makes it an attractive candidate for a position effect.

5.4.2 TUBB4Q, a pseudogene?

The isolation of TUBB4Q by exon trapping

Exon trapping in the FSHD candidate gene region revealed a potential exon 80 kb proximal to D4Z4 with homology to β2-tubulin (van Geel *et al.*, 2000). Genomic sequence analysis and computer alignments resulted in the identification of a putative

transcript with four exons and an ORF of 434 amino acid residues, which was called *TUBB4Q* (GenBank Accession number U83110).

TUBB4Q; a member of the β-tubulin family

TUBB4Q shows 87% sequence homology to β2-tubulin, which is a member of a large tubulin family with copies on many chromosomes. PCR and sequence analysis indeed showed TURB4Q-related sequences on the chromosomes 1, 9, 10, 12, 16, 18 and Y (van Geel *et al.,* 2000). β-tubulins are highly conserved and heterodimers of α- and β-tubulin form the main subunit of microtubules (reviewed by Gundersen (2002)). Microtubules are cylindrical protein structures, which are involved in many processes such as cell division, intracellular transport of vesicles and organelles, cell migration, cell polarity and cell morphology (Kirschner, 1978).

TUBB4Q; the FSHD gene or a pseudogene?

Many non-functional β-tubulin homologues have been described to be intronless pseudogenes or genes with disrupted ORFs (Lee *et al.,* 1983). Despite its evolutionarily constrained ORF, *TUBB4Q* is probably also a pseudogene since high allelic sequence variability was observed, even in coding regions. The most important substitution is the replacement of the initiation codon methionine by a threonine (van Geel *et al.,* 2000). In addition, extensive RT-PCR efforts failed to detect expression in any of the tested tissues (van Geel *et al.,* 2000), suggesting that *TUBB4Q* is indeed a pseudogene.

However, the *TUBB4Q* pseudogene might be ectopically expressed owing to the local chromatin relaxation upon contraction of the D4Z4 repeat. As a result, the non-functional mutated protein can possibly exert a dominant negative effect on functional members of the β-tubulin family and thereby interfere with their function. This would make *TUBB4Q* an ideal candidate gene for FSHD.

5.4.3 FRG2, a muscle-specific transcript

The in silico identification of FRG2

A PAC clone (226K22) proximal to the D4Z4 repeat was used for *in silico* gene predictions by the Genotator browser (Harris, 1997; van Geel *et al.,* 1999). In the region 37 kb proximal to the repeat, four exons were predicted (Rijkers *et al.,* in preparation). Subsequent computer analysis showed a putative muscle-specific promoter including a TATA and a CCAAT box, belonging to the novel transcript, which was called FSHD Region Gene 2 (*FRG2,* GenBank Accession number AY028079).

Primers within each predicted exon were used for RT-PCR analysis on the monochromosomal cell line GM11687, which contains only chromosome 4 as human component. GM11687 was shown to constitutively express *FRG2*. The transcriptional initiation site and the polyadenylation signal were identified by 5′ and 3′ RACE experiments, respectively. The transcript with a length of 2084 base-pairs consists of four exons and encodes a protein of 278 amino acid residues.

Homologues and orthologues

A monochromosomal somatic cell hybrid mapping panel (UK HGMP Resource) was screened by PCR for *FRG2*-related copies, which were found on chromosomes 1, 4, 8, 10, 18 and 20. BLAST searches identified additional copies on chromosomes 3, 7, 16, 19, 21 and 22. The chromosome 10 homologue was shown to be virtually identical to the chromosome 4 copy, with only four amino acid substitutions (Rijkers *et al.,* in preparation). *FRG2* was demonstrated to be primate-specific since no orthologues were found in other species.

Muscle-specific FRG2 expression

Recently, *FRG2* expression of the 4qter locus was detected in FSHD muscle and in differentiating myoblasts (Gabellini *et al.*, 2002; Rijkers *et al.*, in preparation), in addition to the monochromosomal GM11687 cell line. No expression was found in healthy muscle biopsies, in proliferating myoblasts or in peripheral blood lymphocytes (PBL), indicating FSHD muscle-specific transcription. Myoblasts from non-FSHD myopathy patients however showed expression of distantly related *FRG2* copies (Rijkers *et al.,* in preparation). Moreover, *FRG2* up-regulation was demonstrated to be inversely related to the residual D4Z4 repeat size (Gabellini *et al.,* 2002).

Functional analysis of the FRG2 protein

No homology of the *FRG2* protein (FRG2P) with other proteins was found. *In silico,* a putative peroxisomal targeting signal (PST-1) was detected, which is probably non-functional since additional amino acid residues flanking the tripeptide AKL are missing (Hettema *et al.,* 1999). In addition, two nuclear localization signals (NLS) of four basic amino acid residues each were predicted. Subsequent transient transfection experiments with *FRG2* fusion constructs indeed showed a nuclear localization.

The function of FRG2 protein is unknown, but it might be involved in muscle regeneration since forced myogenesis experiments with infection of *MyoD*-expressing adenoviruses in human fibroblasts showed *MyoD*-dependent up-regulation of *FRG2* expression (Rijkers *et al.,* in preparation).

FRG2; the FSHD gene?

FRG2 has been mapped only 37 kb proximal to the D4Z4 repeat. It is postulated that transcriptional regulation of genes closely mapped to a rearrangement is often more affected than that of genes further away (Bedell *et al.,* 1996). Secondly, the transcriptional up-regulation of *FRG2* in differentiating myoblasts and muscle biopsies from FSHD patients indicate a disease- and muscle-specific role. Finally, the *MyoD* induced expression after forced myogenesis suggests a role for *FRG2* in muscle regeneration. However, Lemmers *et al.* described two FSHD families with deletions in the region proximal to the D4Z4 repeat, including the *FRG2* gene (Lemmers *et al.,* 2003). In one of these families, the healthy father carried a normal D4Z4 repeat array but lacked one *FRG2* copy as a consequence of this deletion. His son, affected with FSHD, showed an expansion of the deletion into the D4Z4 repeat, resulting in an array within the FSHD

range. In the second family, the proximally extended D4Z4 deletion including *FRG2* is stably segregating with the affected members. These results challenge a potential role for *FRG2* in FSHD pathogenesis.

5.4.4 ANT1, a muscle-specific mitochondrial protein

ANT1, a bi-functional protein in mitochondria

Adenine Nucleotide Translocator 1 (ANT1) is a highly abundant mitochondrial protein, which is encoded by a transcript of four exons with a length of 893 base-pairs (GenBank Accession number J02966). The gene is mapped as much as 5 Mb proximal to the D4Z4 repeat and is expressed in postmitotic cell types like skeletal muscle, heart and brain (Stepien *et al.,* 1992; Doerner *et al.,* 1997). As a homodimer, ANT1 forms a gated channel to mediate aerobic energy metabolism by exchanging cytosolic ADP for mitochondrial ATP. In addition, ANT1 is involved in mitochondrial-mediated apoptosis as a central component of the mitochondrial permeability transition pore complex (PTPC). During apoptosis, this complex allows free passage of water and ions, causing a mitochondrial membrane potential loss. As a result, pre-apoptotic proteins such as cytochrome *c* and pro-caspases are released from the inner membrane space (reviewed in Belzacq *et al.* (2002)).

ANT1, the FSHD gene?

When *ANT1* was mapped to the 4q35 region (Li *et al.,* 1989), it immediately became an interesting candidate gene for FSHD because of its function and its expression in skeletal muscle cells. Interestingly, two FSHD cases were described, one with mitochondrial myopathy and a FSHD patient with hepatomegaly, who showed a complex III deficiency on the mitochondrial respiratory chain (Hudgson *et al.,* 1972; Slipetz *et al.,* 1991). Subsequent sequence analysis of ANT1 did not reveal any differences between patients and controls (Haraguchi *et al.,* 1993). Recently, ANT1 up-regulation was demonstrated in FSHD muscle consistent with the hypothesis that FSHD is caused by a position effect (Gabellini *et al.,* 2002).

On the one hand, the transcriptional up-regulation, its mitochondrial function and the expression in skeletal muscle cells make *ANT1* an interesting candidate gene for FSHD. On the other hand, up-regulation of *ANT1* as part of the PTPC suggests an increase in apoptotic processes. However, only very low levels of apoptotic cells in the subsarcolemma and a heterogeneous caspase 3 activity were observed in FSHD muscle as compared to Duchenne muscular dystrophy (DMD) muscle (Sandri *et al.,* 2001). In addition, no correlation between the number of apoptotic cells and the clinical phenotype was found in FSHD patients indicating that programmed cell death is not a prominent feature in this disease. These results, in combination with the long distance between *ANT1* and the D4Z4 repeat (5 Mb) make *ANT1* a less attractive candidate gene for FSHD.

5.5 Other candidate genes

Based on the position effect hypothesis, all genes within the immediate vicinity of the D4Z4 repeat may be involved in FSHD. The candidate region might extend to at least 5 Mb proximal to the D4Z4 repeat if the transcriptional up-regulation of *ANT1* observed in FSHD muscle is a direct effect of the contraction of the D4Z4 repeat. The entire region, however, is strikingly gene-poor with a complete lack of coding sequences 250 kb proximal to *FRG1* (van Geel *et al.,* 1999). Detailed sequence analysis proximal to this 250 kb genomic region revealed the presence of some additional genes such as the melatonin receptor *(MTNR1A),* Toll-like receptor 3 *(TLR3)* and *FAT,* but there are as yet no clues about their involvement in the FSHD pathology (Hewitt and Bolland, 2002).

One of the genes studied in more detail is actinin-associated LIM protein *(ALP),* which was found as a muscle-specific transcript by screening a human muscle cDNA array (Bouju *et al.,* 1999). Polyclonal antibodies were used for quantitative Western blotting and immunohistochemical studies, but failed to detect any differences in expression, size or localization of ALP between FSHD patients and controls (Bouju *et al.,* 1999).

Since FSHD seems to be caused by up-regulation (or down-regulation) of genes adjacent to the D4Z4 repeat, microarray experiments may accelerate the search for interesting candidate genes (Chapter 23).

5.6 Concluding remarks

The established transcriptional up-regulation of a few genes in the FSHD region due to a derepression mechanism (Gabellini *et al.*, 2002; see Chapter 10) supports the hypothesis that the disease is caused by deregulation of genes in the vicinity of the D4Z4 repeat. Expression profiling experiments may be performed to study the up- or down-regulation of further genes in the 4q35 region and on other chromosomes (see Chapter 23).

Since the altered gene expression is probably caused by a position effect, it is very unlikely that structural rearrangements (mutations) in one of the candidate genes will underlie FSHD pathogenesis. The transcriptional deregulation of the 4q35 region suggests a complex mechanism in which several genes might be involved; this may well explain the high inter- and intrafamilial phenotypic variability. However, the major hurdle may be discriminating between primarily and secondarily affected genes in the pathogenesis of this muscular dystrophy. Therefore, appropriate animal models may become extremely important.

5.7 References

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6.
Evolution and structural organization of the homeobox-containing repeat D4Z4

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6.1 Introduction

As discussed in other chapters, FSHD is associated with deletions in a complex tandem DNA repeat (D4Z4) on human chromosome 4q35. These deletions are causal for the disease, although the precise underlying mechanism is still unclear. The presence of a double-homeobox motif with each D4Z4 unit is most intriguing and has fuelled much speculation about whether the repeat encodes a protein. However, although some D4Z4-related sequences have been identified in cDNA libraries or amplified by RT-PCR, no *bona fide* transcripts from D4Z4 have ever been reported. In this chapter, I describe the cloning of D4Z4, discuss its structure and give an overview about what is known about its conservation in other organisms and its evolutionary history.

6.2 Organization of the D4Z4 repeat

My laboratory originally identified D4Z4 as part of a screening strategy designed to identify novel homeobox genes. A chromosome 4-specific cosmid library was probed with a degenerate oligonucleotide for homeobox sequences. Detailed mapping and partial sequencing of one of the positive clones (cosmid 13E) showed it to contain a tandem array of DNA repeats, initially defined by a 3.3 kb *Kpn*I fragment and subsequently designated D4Z4 (Wijmenga *et al.*, 1992; van Deutekom *et al.*, 1993; Hewitt *et al.*, 1994). This whole region of chromosome 4q35 has subsequently been found to be packed with low and high copy repeats. Thus, the identification of the p13E-11 probe from cosmid 13E by Cisca Wijmenga in 1992 represented a *tour de force* in cloning. This probe was pivotal in identifying the causative mutation in FSHD (Wijmenga *et al.*, 1992) and is now invaluable for molecular diagnostics.

The organization of the D4Z4 repeat is rather unusual *(Figure 6.1)*, in particular the presence of two homeobox sequences within the same open reading frame. Homeobox genes, encoding homeodomain transcription factors, often play important roles in embryonic development. The predicted homeodomains from D4Z4 (which share 52% amino acid identity) are both most closely related to the Mix and paired families. Because of the potential of D4Z4 to encode a protein, several groups have focused on searching for transcripts from D4Z4. Although cDNAs and RT-PCR products containing closely related sequences have been identified, none originate from chromosome 4q35. For example, we isolated several related cDNA clones representing transcripts from acrocentric chromosomes. However, all of these cDNAs contained in-frame stop codons within the predicted homeodomains (Hewitt *et al.*, 1994; Lyle *et al.*, 1995). Subsequently, clones that could encode such double-homeodomain proteins have been identified; again these do not represent transcripts from D4Z4 but rather are from other loci (Ding *et al.*, 1998; Gabriels *et al.*, 1999; Beckers *et al.*, 2001). Thus, there is currently no conclusive evidence that D4Z4 is transcribed.

Within each D4Z4 repeat, the homeoboxes are flanked by two classes of repetitive DNA; a GC-rich low copy repeat originally named hhspm3 (Zhang *et al.*, 1987) and LSau (Agresti *et al.*, 1987, 1989; Meneveri *et al.*, 1993). LSau repeats are dispersed, preferentially found in regions of heterochromatin and are often associated with 68 bp (or β) satellite DNA (Meneveri *et al.*, 1993). On chromosome 4q35, 68 bp satellite DNA is not interspersed between repeat units, but present as a block of about 8 kb immediately distal to D4Z4 (van Geel *et al.*, 2002a). This satellite is only present on one variant (4qA) of the 4q telomere.

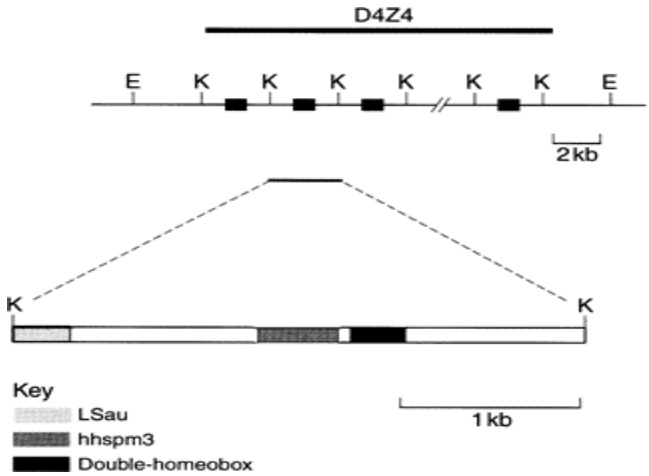


Figure 6.1 A schematic diagram of the D4Z4 repeat. D4Z4 is contained within a polymorphic *Eco*RI fragment. Each copy of the 3.3 kb repeat contains a single *Kpn*I site. Each D4Z4 unit

contains a double homeobox sequence (black box) as well as LSau and hhspm3 repeats (hatched boxes). E, *Eco*RI site; K, *Kpn*I site.

The D4Z4 repeat units are remarkably homogeneous, with intra-array differences of <0.5%. Although rare, there is a precedent for human protein-coding genes to be arranged in long tandem arrays. The *TSPY* locus on the Y chromosome (Manz *et al.*, 1993), is probably the best described example. Sequencing of the Y chromosome from a single individual identified 35 *TSPY* repeats (each being 20.4 kb) in a single, homogeneous array with the units rarely differing from the consensus by more than 1% (Skaletsky *et al.*, 2003). *TSPY* is transcribed, although the function of the protein product is unknown. By analogy, a functional role for D4Z4 as a transcription unit should not be completely ruled out.

6.3 D4Z4-related sequences in the human genome

From initial fluorescent *in situ* hybridization (FISH) and Southern blot studies, it was apparent that D4Z4 is part of a dispersed repeat family, with related sequences on many other chromosomes. Most strikingly, there is a closely related tandem array on chromosome 10qter that has 98% nucleotide identity to D4Z4 (Deidda *et al.*, 1995; van Geel *et al.*, 2002a). In addition, there are numerous 3.3 kb repeat-like sequences on many other chromosomes, including the pericentromeric regions of the acrocentric chromosomes (Lyle *et al.*, 1995), chromosome Y and chromosome 3p (Ballarati *et al.*, 2002). These show a much lower level of sequence homology to D4Z4 (92–97% nucleotide identity) than does the 10q26 array. Furthermore, in contrast to the ordered tandem arrays on chromosomes 4q35 and 10q26, these more divergent copies are interspersed with β satellite DNA and other repeat sequences including satellite 2 and 3 (Lyle, 1995; Lyle *et al.*, 1995; Ballarati *et al.*, 2002).

With the completion of the euchromatic portion of the human genome and partial sequence of heterochromatic regions, some of these dispersed copies can now be examined in more detail. Ballaratri *et al.* (2002) sequenced a PAC clone from chromosome 22p and showed it to contain three copies of a D4Z4-like repeat interspersed with β satellite DNA, confirming previous YAC and fibre-FISH analyses of acrocentric 3.3 kb repeat loci (Lyle *et al.*, 1995; Winokur *et al.*, 1996). Bioinformatic analysis of a BAC clone mapping to chromosome 3 (RP11–413E6; AC108724) shows it to contain one D4Z4-like element flanked by LSau and β satellite DNA (unpublished data); thus, the 3.3 kb repeats are not always present as arrays. The sequence of an unmapped Y chromosome BAC (RP11–886I11) has been deposited in GenBank under Accession Number AC134882. Examination of this sequence shows it to contain four copies of a D4Z4-like sequence interspersed with variable amounts of β satellite DNA (ranging from 2–8 kb). As most heterochromatic regions (where 3.3 kb repeats are preferentially localized) have not yet been sequenced, a detailed understanding of the organization of, and relationships between, the 3.3 kb loci is not yet possible. However, it is clear that the

organization of D4Z4-related sequences is very complex and variable, indicating a complex evolutionary history with a large number of duplication and rearrangement events.

The distal 1Mb region of human chromosome 4q35 is part of a complex duplicon with related sequences on many human chromosomes (van Geel *et al.,* 1999, 2002a). Although in some cases these 4q35 duplicons are also associated with 3.3 kb loci, they do not always co-localize (Ballarati *et al.,* 2002; van Geel *et al.,* 2002b). Furthermore, polymorphisms in the sequence content of some of these 4q35 duplications have been observed (Piccini *et al.,* 2001). Such plasticity is a common property of the human genome (Bailey *et al.,* 2002) and the evolutionary history of the chromosome 4q35 region is likely to be complex.

6.4 Conservation of D4Z4 in other organisms

In the early 1990s, we and others used genomic library screening and degenerate PCR to try to identify homologous D4Z4 sequences in other organisms. On Southern blots, the only species for which we saw reproducible cross-hybridization signals with D4Z4 probes, were primates (Hewitt *et al.,* 1994; Clark *et al.,* 1996). Library screening at reduced stringency and the use of several degenerate PCR strategies all failed to identify any orthologous sequences from other vertebrates, including mouse and chick (Clark, 1995). However, investigation of D4Z4 repeat homologues in primates, in particular the apes, proved more fruitful. Southern blot and FISH experiments were used to investigate copy number and the chromosomal distribution of the 3.3 kb repeat family in a number of primates (Clark, 1995; Clark *et al.,* 1996).

6.4.1 D4Z4 orthologues in primates

In the chimpanzee *(Pan troglodytes),* gorilla and orang-utan, distinctive 3.3 kb *Kpn*I fragments on Southern blots suggested the presence of similar repeat arrays in the great apes (Clark *et al.,* 1996). There were additional, less intense cross-hybridizing fragments on Southern blots, particularly in chimpanzee and orang-utan, indicating that the great ape genomes also contain D4Z4-related sequences. More detailed Southern analysis using a range of probes and restriction enzymes showed very similar hybridization patterns for all the sequence elements that make up the D4Z4 repeat (Clark *et al.,* 1996). Furthermore, analysis of genomic DNA isolated from lymphoblastoid cell lines by pulsed-field gel electrophoresis showed that most of these D4Z4-related sequences are arranged in large arrays. For some of the great ape species we could also infer polymorphisms in array number (Clark *et al.,* 1996).

The association of the 3.3 kb repeat family with the telomere of chromosome 4q is also conserved in the great apes. Using FISH, D4Z4 probes cross-hybridize to the long arm of chimpanzee, gorilla and orang-utan chromosome III—the homologue of human chromosome 4 (Clark *et al.,* 1996; Winokur *et al.,* 1996). In addition, there are dispersed FISH signals on the short arms or pericentromeric regions of the ape acrocentric chromosomes; many of these 3.3 kb repeat loci co-localize with rDNA genes and/or β satellite DNA (Clark, 1995). The FISH and Southern blot data are consistent with and

suggestive of, a D4Z4-like array and additional dispersed 3.3 kb repeat sequences in the apes.

In more distantly related primates, such as Old World monkeys, the hybridization complexity on Southern blots is significantly reduced (Clark *et al.*, 1996; Winokur *et al.*, 1996). Pulsed-field gel electrophoresis of rhesus macaque DNA showed these loci to be predominantly arranged in large (>200 kb) polymorphic arrays. Using FISH, two telomeric loci were identified, one of which was shown to correspond to human chromosome 4q by chromosome painting (Clark *et al.*, 1996). Although most extensive data are from rhesus macaque, Southern blot and FISH analyses of two other species of Old World monkey (baboon and crab-eating macaque) and a New World monkey (marmoset) are consistent with the finding of reduced 3.3 kb repeat loci in more distant primates (Clark *et al.*, 1996).

A more recent study has looked at the evolutionary relationship between the D4Z4 repeat and the wider 4q35 domain in primates using FISH (Ballarati *et al.*, 2002). Multiple 4q35 paralogous sequences were observed in great ape, gibbon and baboon, indicating that an extensive duplication of chromosome 4q35 occurred at least 25 million years ago, before the split of the great ape and Old World monkey lineages. At least some of these duplication events probably occurred independently of D4Z4 amplification as our data suggest significant amplification and dispersion of 3.3 kb repeat loci after the divergence of these lineages.

6.4.2 Further analysis of the evolution of D4Z4; the potential of genome sequence projects

In 1995, we screened a rhesus macaque cosmid library and isolated several clones containing sequences similar to D4Z4. Partial sequencing of these cosmids identified D4Z4-like sequences (see GenBank Accession nos. U89918, U89920, U89921). However, although clustered, these sequences are not arranged in tandem arrays and probably represent dispersed 3.3 kb repeats, rather than D4Z4 orthologues (Clark, 1995). Winokur *et al.* (1996) also reported the partial sequence of these macaque D4Z4-related sequences.

At that time, the lack of readily available large insert libraries for non-human species made it difficult to investigate the organization of D4Z4 homologues in other primates. With the completion of the human genome sequence, there has been a drive for comparable projects in several non-human primates, most notably the chimpanzee (McConkey and Varki, 2000; Varki, 2000), and the output from these projects may prove very useful for the analysis of D4Z4 evolution.

A shotgun sequencing strategy is being employed to sequence the chimpanzee genome. DNA has been isolated from several individuals to generate 3–4X genome coverage (www.hgsc.bcm.tmc.edu/). For most regions of the genome this approach is highly efficient; given the high degree of sequence identity between human and chimpanzee, many of the shotgun reads can be aligned directly onto the human sequence. However, the shotgun approach used leads to problems in assembly of tandem repeat arrays. We had problems in assembly of shotgun sequence from a human BAC clone containing multiple copies of 10q26 3.3 kb repeats (van Geel *et al.*, 2002a). A detailed comparison of D4Z4 sequence between human and chimpanzee will require detailed

physical maps to be assembled and anchored to orthologous single-copy sequences. However, it has been possible to assemble a consensus chimpanzee D4Z4 repeat sequence and this is shown in *Figure 6.2*.

A human D4Z4 sequence (GenBank Accession number AF1 17653) was used to search the shotgun reads from the chimpanzee genome project using megaBLAST (www.ncbi.nlm.nih.gov/blast/tracemb.html). Fifty-one traces with matches >97% identity were downloaded, then aligned and edited using Sequencher (GeneCodes, USA). This allowed assembly of a consensus 3.3 kb repeat with high similarity to

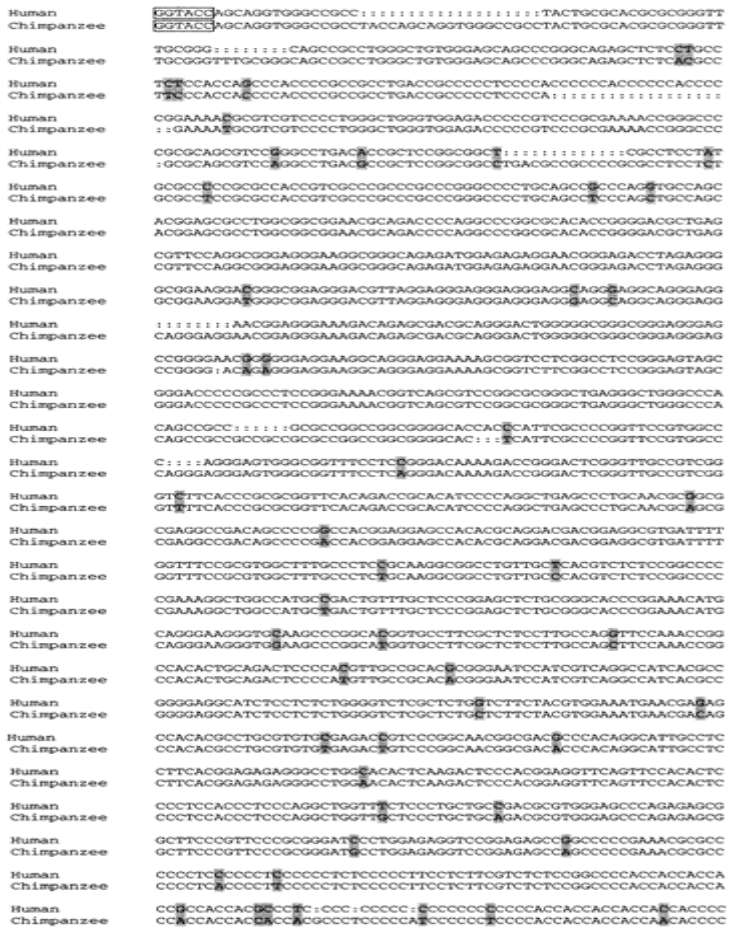
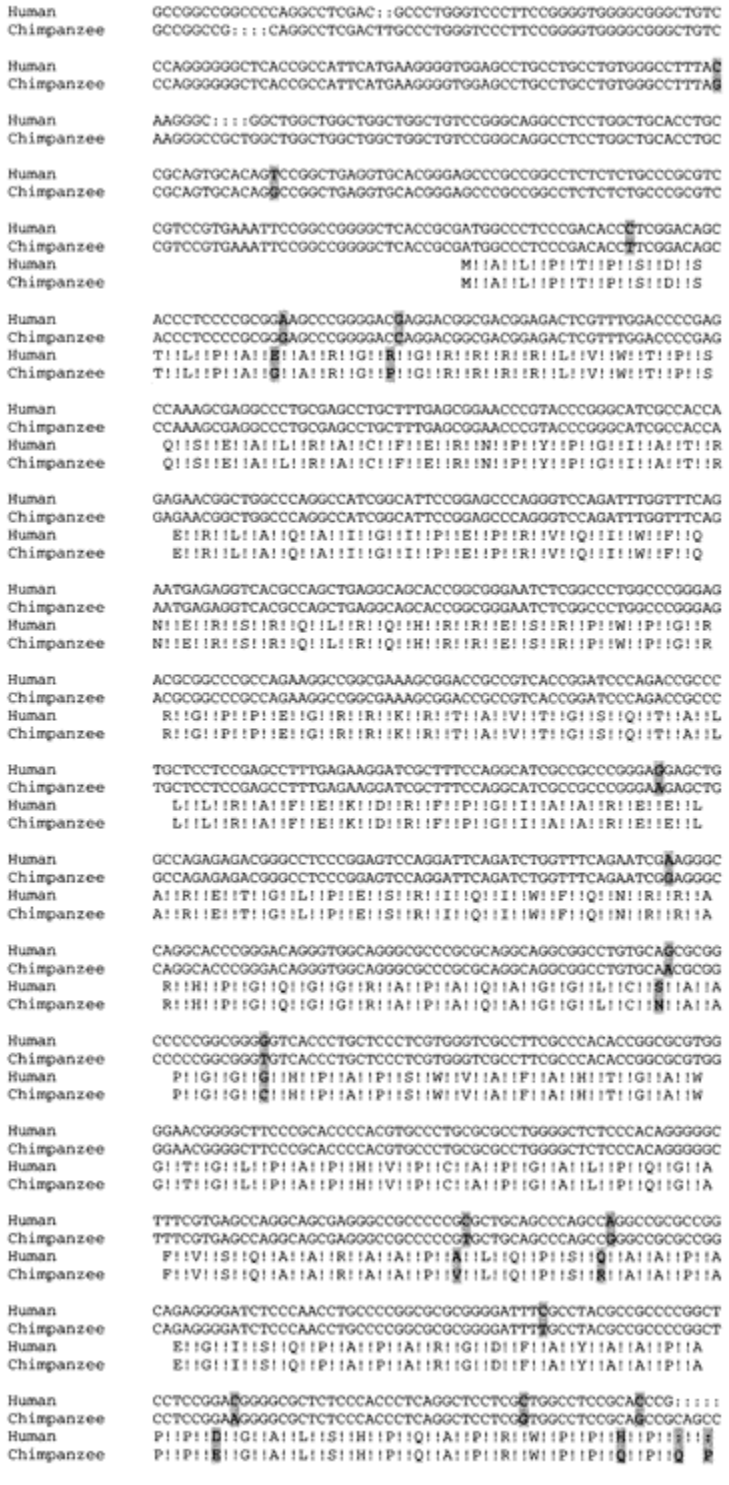


Figure 6.2 Alignment of D4Z4 sequences from human and chimpanzee. The human sequence is from Gabriels *et al.* (1999), GenBank Accession no. AF1 17653. The

chimpanzee sequence is a consensus compiled from shotgun sequences identified using cross-species megaBLAST (www.ncbi.nlm.nih.gov/blast/tracemb.html). The repeat is defined by the *Kpn*I site (boxed). Gaps are indicated by a colon (:). Nucleotide differences are shaded. The conceptual translations of the human and chimpanzee homeodomain-containing open reading frames are shown under the DNA alignment.


Human GCCGGCCGGCCCCAGGCCTCGAC::GCCCTGGGTCCCTTCCGGGGTGGGGCGGGCTGTC
Chimpanzee GCCGGCCG::::CAGGCCTCGACTTGCCCTGGGTCCCTTCCGGGGTGGGGCGGGCTGTC
Human CCAGGGGGGCTCACCGCCATTCATGAAGGGGTGGAGCCTGCCTGCCTGTGGGCCTTTAC
Chimpanzee CCAGGGGGGCTCACCGCCATTCATGAAGGGGTGGAGCCTGCCTGCCTGTGGGCCTTTAG
Human AAGGGC::::GGCTGGCTGGCTGGCTGGCTGTCCGGGCAGGCCTCCTGGCTGCACCTGC
Chimpanzee AAGGGCCGCTGGCTGGCTGGCTGGCTGTCCGGGCAGGCCTCCTGGCTGCACCTGC
Human CGCAGTGCACAGTCCGGCTGAGGTGCACGGGAGCCCGCCGGCCTCTCTCTGCCCGCGTC
Chimpanzee CGCAGTGCACAGGCCGGCTGAGGTGCACGGGAGCCCGCCGGCCTCTCTCTGCCCGCGTC
Human CGTCCGTGAAATTCCGGCCGGGGCTCACCGCGATGGCCCTCCCGACACCCTCGGACAGC
Chimpanzee CGTCCGTGAAATTCCGGCCGGGGCTCACCGCGATGGCCCTCCCGACACCTTCGGACAGC
Human M!!A!!L!!P!!T!!P!!S!!D!!S
Chimpanzee M!!A!!L!!P!!T!!P!!S!!D!!S
Human ACCCTCCCCGCGGAAGCCCGGGGACGAGGACGGCGACGGAGACTCGTTTGGACCCCGAG
Chimpanzee ACCCTCCCCGCGGAGCCCGGGGACCAGGACGGCGACGGAGACTCGTTTGGACCCCGAG
Human T!!L!!P!!A!!E!!A!!R!!G!!R!!G!!R!!R!!R!!R!!L!!V!!W!!T!!P!!S
Chimpanzee T!!L!!P!!A!!G!!A!!R!!G!!P!!G!!R!!R!!R!!R!!L!!V!!W!!T!!P!!S
Human CCAAAGCGAGGCCCTGCGAGCCTGCTTTGAGCGGAACCCGTACCCGGGCATCGCCACCA
Chimpanzee CCAAAGCGAGGCCCTGCGAGCCTGCTTTGAGCGGAACCCGTACCCGGGCATCGCCACCA
Human Q!!S!!E!!A!!L!!R!!A!!C!!F!!E!!R!!N!!P!!Y!!P!!G!!I!!A!!T!!R
Chimpanzee Q!!S!!E!!A!!L!!R!!A!!C!!F!!E!!R!!N!!P!!Y!!P!!G!!I!!A!!T!!R
Human GAGAACGGCTGGCCCAGGCCATCGGCATTCCGGAGCCCAGGGTCCAGATTTGGTTTCAG
Chimpanzee GAGAACGGCTGGCCCAGGCCATCGGCATTCCGGAGCCCAGGGTCCAGATTTGGTTTCAG
Human E!!R!!L!!A!!Q!!A!!I!!G!!I!!P!!E!!R!!V!!Q!!I!!W!!F!!Q
Chimpanzee E!!R!!L!!A!!Q!!A!!I!!G!!I!!P!!E!!R!!V!!Q!!I!!W!!F!!Q
Human AATGAGAGGTCACGCCAGCTGAGGCAGCACCGGCGGGAATCTCGGCCCTGGCCCGGGAG
Chimpanzee AATGAGAGGTCACGCCAGCTGAGGCAGCACCGGCGGGAATCTCGGCCCTGGCCCGGGAG
Human N!!E!!R!!S!!R!!Q!!L!!R!!Q!!H!!R!!R!!E!!S!!R!!P!!W!!P!!G!!R
Chimpanzee N!!E!!R!!S!!R!!Q!!L!!R!!Q!!H!!R!!R!!E!!S!!R!!P!!W!!P!!G!!R
Human ACGCGGCCCGCCAGAAGGCCGGCGAAAGCGGACCGCCGTCACCGGATCCCAGACCGCCC
Chimpanzee ACGCGGCCCGCCAGAAGGCCGGCGAAAGCGGACCGCCGTCACCGGATCCCAGACCGCCC
Human R!!G!!P!!P!!E!!G!!R!!R!!K!!R!!T!!A!!V!!T!!G!!S!!Q!!T!!A!!L
Chimpanzee R!!G!!P!!P!!E!!G!!R!!R!!K!!R!!T!!A!!V!!T!!G!!S!!Q!!T!!A!!L
Human TGCTCCTCCGAGCCTTTGAGAAGGATCGCTTTCCAGGCATCGCCGCCCGGGAGGAGCTG
Chimpanzee TGCTCCTCCGAGCCTTTGAGAAGGATCGCTTTCCAGGCATCGCCGCCCGGGAGGAGCTG
Human L!!L!!R!!A!!F!!E!!K!!D!!R!!F!!P!!G!!I!!A!!A!!R!!E!!E!!L
Chimpanzee L!!L!!R!!A!!F!!E!!K!!D!!R!!F!!P!!G!!I!!A!!A!!R!!E!!E!!L
Human GCCAGAGAGACGGGCCTCCCGGAGTCCAGGATTCAGATCTGGTTTCAGAATCGAAGGGC
Chimpanzee GCCAGAGAGACGGGCCTCCCGGAGTCCAGGATTCAGATCTGGTTTCAGAATCGGAGGGC
Human A!!R!!E!!T!!G!!L!!P!!E!!S!!R!!I!!Q!!I!!W!!F!!Q!!N!!R!!R!!A
Chimpanzee A!!R!!E!!T!!G!!L!!P!!E!!S!!R!!I!!Q!!I!!W!!F!!Q!!N!!R!!R!!A
Human CAGGCACCCGGGACAGGGTGGCAGGGCGCCCGCGCAGGCAGGCGGCCTGTGCAGCGCGG
Chimpanzee CAGGCACCCGGGACAGGGTGGCAGGGCGCCCGCGCAGGCAGGCGGCCTGTGCAACGCGG
Human R!!H!!P!!G!!Q!!G!!G!!R!!A!!P!!A!!Q!!A!!G!!G!!L!!C!!S!!A!!A
Chimpanzee R!!H!!P!!G!!Q!!G!!G!!R!!A!!P!!A!!Q!!A!!G!!G!!L!!C!!N!!A!!A
Human CCCCCGGCGGGGGTCACCCTGCTCCCTCGTGGGTCGCCTTCGCCCACACCGGCGCGTGG
Chimpanzee CCCCCGGCGGGTGTCACCCTGCTCCCTCGTGGGTCGCCTTCGCCCACACCGGCGCGTGG
Human P!!G!!G!!G!!H!!P!!A!!P!!S!!W!!V!!A!!F!!A!!H!!T!!G!!A!!W
Chimpanzee P!!G!!G!!C!!H!!P!!A!!P!!S!!W!!V!!A!!F!!A!!H!!T!!G!!A!!W
Human GGAACGGGGCTTCCCGCACCCCACGTGCCCTGCGCGCCTGGGGCTCTCCCACAGGGGGC
Chimpanzee GGAACGGGGCTTCCCGCACCCCACGTGCCCTGCGCGCCTGGGGCTCTCCCACAGGGGGC
Human G!!T!!G!!L!!P!!A!!P!!H!!V!!P!!C!!A!!P!!G!!A!!L!!P!!Q!!G!!A
Chimpanzee G!!T!!G!!L!!P!!A!!P!!H!!V!!P!!C!!A!!P!!G!!A!!L!!P!!Q!!G!!A
Human TTTCGTGAGCCAGGCAGCGAGGGCCGCCCCCGCGCTGCAGCCCAGCCAGGCCGCGCCGG
Chimpanzee TTTCGTGAGCCAGGCAGCGAGGGCCGCCCCCGTGCTGCAGCCCAGCCGGGCCGCGCCGG
Human F!!V!!S!!Q!!A!!A!!R!!A!!A!!P!!A!!L!!Q!!P!!S!!Q!!A!!A!!P!!A
Chimpanzee F!!V!!S!!Q!!A!!A!!R!!A!!A!!P!!V!!L!!Q!!P!!S!!R!!A!!A!!P!!A
Human CAGAGGGGATCTCCCAACCTGCCCCGGCGCGCGGGGATTTCGCCTACGCCGCCCCGGCT
Chimpanzee CAGAGGGGATCTCCCAACCTGCCCCGGCGCGCGGGGATTTTGCCTACGCCGCCCCGGCT
Human E!!G!!I!!S!!Q!!P!!A!!P!!A!!R!!G!!D!!F!!A!!Y!!A!!A!!P!!A
Chimpanzee E!!G!!I!!S!!Q!!P!!A!!P!!A!!R!!G!!D!!F!!A!!Y!!A!!A!!P!!A
Human CCTCCGGACGGGGCGCTCTCCCACCCTCAGGCTCCTCGCTGGCCTCCGCACCCG:::::
Chimpanzee CCTCCGGAAGGGGCGCTCTCCCACCCTCAGGCTCCTCGGTGGCCTCCGCAGCCGCAGCC
Human P!!P!!D!!G!!A!!L!!S!!H!!P!!Q!!A!!P!!R!!W!!P!!P!!R!!P!!::::
Chimpanzee P!!P!!S!!G!!A!!L!!S!!H!!P!!Q!!A!!P!!R!!W!!P!!P!!Q!!P!!Q P

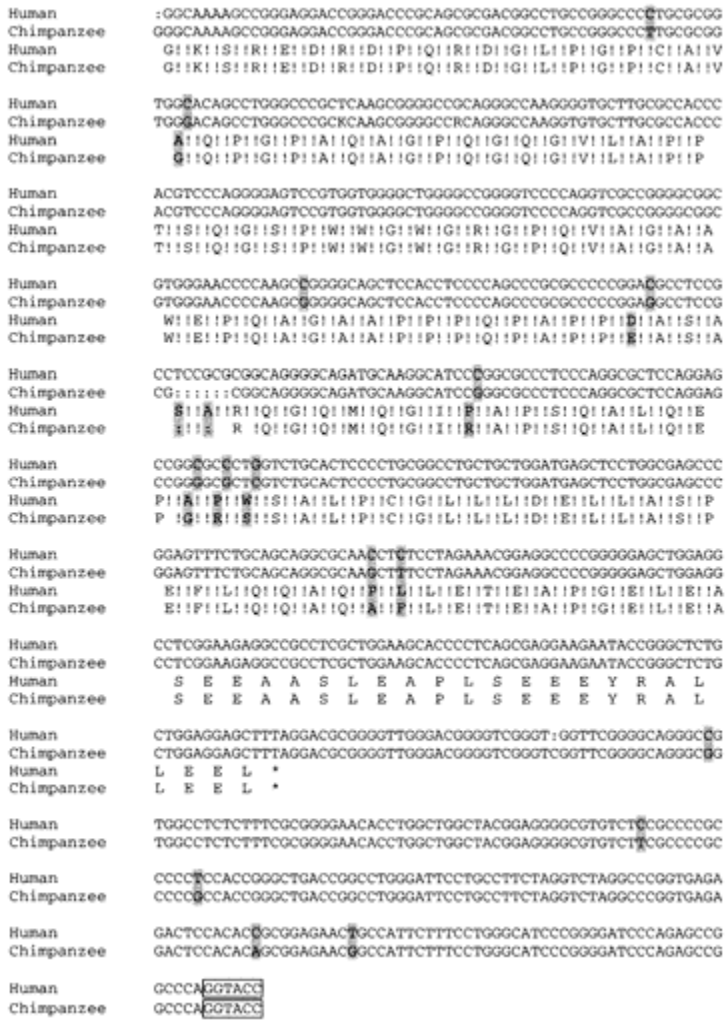


Figure 6.2—

D4Z4 *(Figure 6.2)*. Several nucleotide differences were found between mate-pairs,which typically are reads from each end of a 4 kb clone, indicating intra-array variation. In addition, there may also be variation between chromosomal loci and/orbetween the individuals used to generate the shotgun clones. Therefore, it is notpossible at present to assign any sequences as orthologous to D4Z4.

However, several conclusions may be drawn from examination of the consensus sequence. The chimpanzee repeat unit is approximately 3.3 kb, in accordance with the Southern blot data. As in human, there is a single *Kpn*I site and no *Eco*RI site within each

repeat unit. There is an open reading frame that could encode two homeodomains. *Figure 6.2* shows an alignment of the human DNA sequence and the deduced protein sequence from Gabriels *et al.* (1999) to the chimpanzee consensus. The overall nucleotide identity between human and chimpanzee is 98%. There are 23 nucleotide substitutions within the open reading frame; surprisingly the majority of these (16) are non-synonymous substitutions. For protein coding regions, synonymous substitution usually occurs much faster than non-synonymous substitution. However, a formal analysis of mutation rates of the D4Z4 repeat will require the orthologous sequences to be compared and this is not yet possible.

6.5 Conclusions

The D4Z4 repeat remains something of an enigma. The presence of an open reading frame potentially encoding a homeodomain protein indicates that this repeat was derived by duplication of an ancestral homeobox gene. Whether D4Z4 still has a functional role as a protein-encoding gene is unknown. The repeat apparently binds a repressor complex (Gabellini *et al.*, 2002) and may play a role in establishing a silenced chromosomal domain. However, this model does not explain why an open reading frame through the two potential homeodomains has been maintained. The apparent lack of conservation of the D4Z4 repeat in other vertebrates has made it difficult to identify functional regions, for example by looking at nucleotide divergence. Here, I have identified chimpanzee sequences homologous to D4Z4 and, interestingly, the open reading frame does appear to be conserved. However, because of the shotgun strategy being used for assembly of the chimpanzee genome sequence, unambiguous identification of the orthologous locus may prove difficult. As other primate sequence projects progress, it will become possible to identify homologous sequences in these genomes. Analysis of lower primates, where the D4Z4 repeat family appears to be less complex, may be especially useful for studying the evolutionary history of this remarkable DNA sequence. I hope that by understanding its origins, we may be able to understand more about its functions and relationship to FSHD.

Acknowledgements

I would like to thank all the past and present members of my group who have contributed to our understanding of the role of the D4Z4 repeat in FSHD. With respect to the evolution and distribution of the 3.3 kb repeat family, particular thanks go to Lorraine Clarke (who expended much effort trying to identify homologues of D4Z4 in other organisms) and Robert Lyle. I would also like to thank the Leiden Group headed by Rune Frants and Silvére van der Maarel for fruitful collaboration. Funding for aspects of this work from the MDA USA, Muscular Dystrophy Campaign of Great Britain, the Medical Research Council, the Wellcome Trust, the FSH Society Inc. and the Princes Beatrix Fonds is gratefully acknowledged.

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7.
Subtelomeric exchange between 4q and 10q sequences

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

7.1 Introduction

After the FSHD locus had been mapped to the subtelomeric region of the long arm of chromosome 4 (Wijmenga *et al.,* 1990), the isolation of probe p13-E11 from a cosmid containing homeodomain sequences was the next crucial step in the molecular dissection of the genetic background. Compared to other probes, p13-E11 was almost single copy on Southern blots and highly useful for diagnostic purposes because it usually detected an *Eco*RI fragment shorter than approximately 40 kb in patients (Wijmenga *et al.,* 1992). However, these gels contained several larger fragments, insufficiently resolved by conventional agarose electrophoresis. The parental cosmid clone showed cross-hybridization to several other chromosomes on FISH analysis, in particular to subtelomeric (and pericentromeric) regions (Hewitt *et al.,* 1994), suggesting a complex molecular and evolutionary past.

With the aid of pulsed-field gel electrophoresis (PFGE), the longer *Eco*RI fragments, contained in the compression zone of a conventional agarose gel, could be resolved and proved to be in the range of several hundred kb (Wijmenga *et al.,* 1994; van Deutekom *et al.,* 1996). The extensive interindividual size variation reflected a complex polymorphism. Interestingly, most individuals displayed four fragments. Studies in families indicated the four fragments to reflect alleles at two unlinked loci.

7.2 The second p13E-11 locus maps to 10q26

Multigeneration CEPH (Centre d'Etude du Polymorphisme Humain) families, in which short fragments segregated, were identified for linkage analysis in order to map the two predicted loci. Using microsatellite markers from the candidate regions with FISH cross-

hybridization, chromosomes 4q and 10q were identified as harbouring the two segregating loci (Bakker *et al.,* 1995). Deidda *et al.* (1995) independently confirmed this localization by FISH analysis. This finding had important implications for improving diagnostic reliability. Families had been identified in which short fragments did not segregate with the disease. The occurrence of phenocopies, patients with the syndrome but not the pathognomonic genotype, compromises reliable genetic counselling. Whether these individuals were recombinants or phenocopies could be resolved by haplotyping. Moreover, identification of the chromosome 10q locus had further implications for FSHD. The possible involvement of this locus in non-4q-linked families was excluded by linkage analysis by ourselves and others (Bakker *et al.,* 1995; Spear *et al.,* personal communications). The pressing question was: What is the functional difference between the chromosome 4q and chromosome 10q subtelomeres as only the chromosome 4q locus is associated with FSHD? To allow studies on the role of the two loci, differences needed to be identified.

7.3 Differential detection of the two loci

The chromosome 10q locus apparently showed high sequence homology with the 4q locus and very likely a common evolutionary background. Efforts to substantiate these predictions were undertaken in various laboratories: cloning, physical mapping, and ultimately sequencing of the two regions (Mills *et al.,* 1992; Wijmenga *et al.,* 1993; Wright *et al.,* 1993). Detailed physical maps were generated, clearly showing the same general structure of the two loci. The *Eco*RI fragments basically consist of tandem arrays of 3.3 kb *Kpn*I repeats, with the p13E-11 probe sequence on the proximal edge (van Deutekom *et al.,* 1993). The sequence homology between the *Kpn*I repeats is very high. The group of Dr. L.Felicetti in Rome was able to identify a number of significant sequence differences (Deidda *et al.,* 1996) the most useful of which was a polymorphism that created a *Bln*I site on the chromosome 10q repeat (Deidda *et al.,* 1996). The implications of this finding for diagnosis were obvious. It allows the direct digestion of the interfering chromosome 10q fragments in the fraction of families with complex fragment patterns, and allows the evidence of haplotyping, which is strongly dependent on family structure and marker informativity. The diagnostic implications are discussed in Chapter 15.

Subsequent scrutinization of the chromosome 10- and chromosome 4-derived sequences, identified a restriction site for *Xap*I on chromosome 4. Southern analysis of well-characterized families revealed the expected and complementary fragments seen with *Bln*I (Lemmers *et al.,* 2001).

7.4 ‘Cross-talk’ between the subtelomeres of 4q and 10q

In combination with PFGE, the differential digestion technique opened new perspectives for the molecular and evolutionary analysis of the two loci. When the *Bln*I association to chromosome 10 is complete, PFGE analysis reveals four fragments on *Eco*RI and only two fragments after double digestion with *Eco*RI and *Bln*I (E/B). This general prediction

was indeed borne out in the majority of cases. Interestingly, a much more complex picture emerged when we analysed larger groups of patients and controls *(Figure 7.1)*. In some samples, three or one p13E-11-positive fragment; in rare cases none or four fragments were observed. These results suggested that 4-type arrays could also be present on chromosome 10 and *vice versa* (van Deutekom *et al.*, 1996).

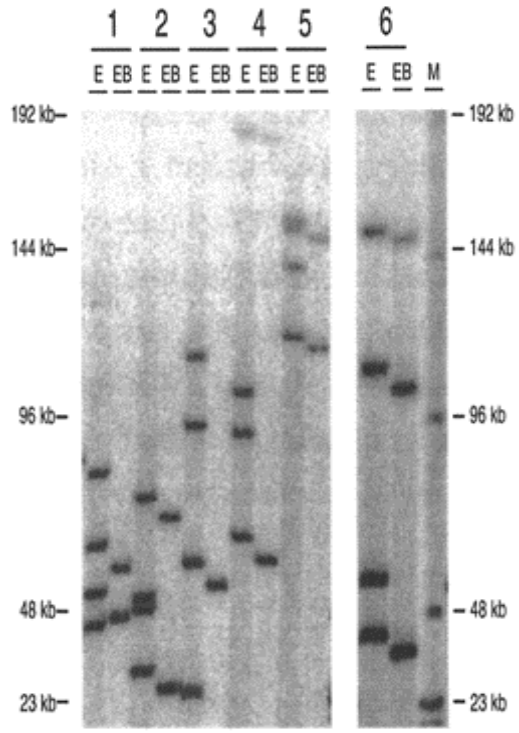

Figure 7.1 Differential restriction analysis of six of 50 unaffected male control individuals. Pulsed-field gel electrophoresis of *Eco*RI (E) and *Eco*RI/*Bln*I (EB) digested DNA and subsequent Southern blot analysis using probe p13E-11. Control 3 appears to be 'monosomic', whereas control 6 appears to be 'trisomic'. Although control 2 is unaffected, he exhibits a short (28 kb), *Bln*I-resistant *Eco*RI fragment. Presumably, this short fragment is linked to

chromosome 10q26. Since control 2 is 'disomic', he probably carries another hybrid fragment that is linked to chromosome 4q35. The marker (M) is a 48 kb phage lambda DNA ladder.

To illustrate this and to prove the interchromosomal exchange model, Fam 28 is discussed in some detail (van Deutekom *et al.*, 1996). In this family, an *Eco*RI fragment of 25 kb (22 after *Bln*I digestion) co-segregates with FSHD, in line with a conventional FSHD1 pedigree *(Figure 7.2)*. Interestingly, the father carries a second short, 22 kb, *Bln*I-resistant fragment. Since the father transmits two chromosome 4 and two chromosome 10 fragments to his children, the 22 kb fragment probably represents somatic mosaicism. Moreover, the father showed two additional *Bln*I-resistant, 4q35-like fragments. Two of his sons also possessed three BlnI-resistant fragments, whilst the youngest son exhibited only one *Bln*I-resistant fragment. The unaffected mother also displayed only one *Bln*I-resistant fragment. Subsequent PFGE analysis fully supported a hypothesis according to which exchange of subtelomeric 3.3 kb repeat-containing fragments between chromosome 4 and chromosome 10 had occurred; these 'trisomic' cases carry two authentic chromosomes 4, while one of the chromosomes 10 carries a 4q35-like structure at the telomere, thereby confounding the *Bln*I test. The 'monosomic' cases show the reciprocal

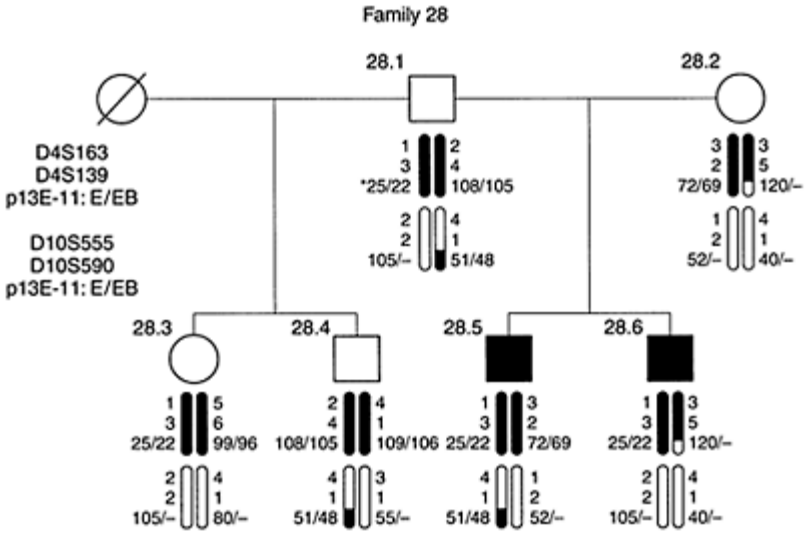

Figure 7.2 Haplotype analysis of family 28 using chromosome 4q35 and 10q26 markers. The sizes of the p13E-11 fragments after *Eco*RI (E) and

*Eco*RI/*Bln*I (EB) are shown. *Eco*RI fragments containing repeated units derived from chromosome 10q26 are completely digested by *Bln*I (−). Haplotypes including hybrid p13E-11 *Eco*RI fragments are depicted by filled (chromosome 4q35) or open (chromosome 10q26) bars with swopped ends. The asterisk indicates that the father (28.1) is mosaic, carrying an additional 22 kb fragment possibly derived from the 25 kb fragment (b).

constitution; one of the chromosomes 4 carries a 10q26-like telomere. From this family, a model emerged for the subtelomeric exchanges between chromosomes 4 and 10 and the consequences for FSHD *(Figure 7.3)*. Haplotype analysis in the family with microsatellite markers mapping just proximal of the p13E-11 probe provides full support to the interchromosomal exchange hypothesis (van Deutekom *et al.*, 1996).

7.5 Prevalence of interchromosomal exchange

For an initial estimate of the frequency of the interchromosomal exchange, a small random population sample of 50 Dutch males was investigated after double digestion with *Eco*RI and *Bln*I and PFGE (van Deutekom *et al.*, 1996). In five cases, trisomy was observed and in another five cases, monosomy was encountered, giving an approximate estimate of 20% (10/50).

The high degree of homology between the chromosome 4 and chromosome 10 subtelomeric regions indicates some kind of interchromosomal cross-talk as the most likely mechanism. Two types of rearrangement can explain the finding; either translocations including the complete subtelomere, or gene conversion. In the latter case it is more likely that the flanking subtelomeric sequences are not involved.

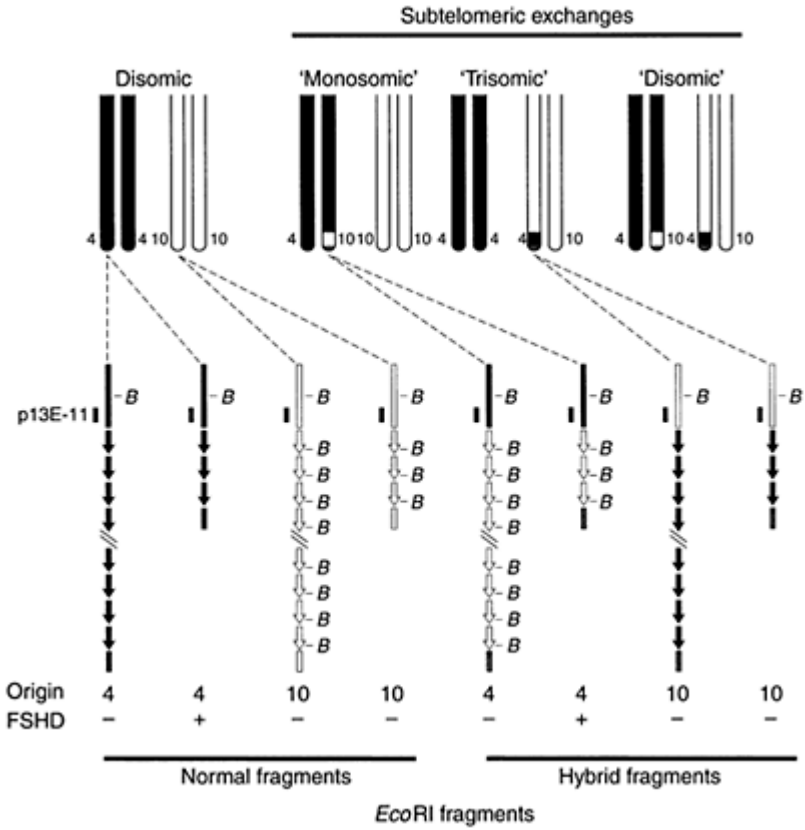

Figure 7.3 Model to explain the aberrant *Bln*I restriction patterns observed after differential restriction analysis. These patterns suggest some cases to be 'monosomic' or 'trisomic' for the chromosome 4q35 *Eco*RI fragment. On top, the subtelomeric regions of chromosomes 4q35 (filled bars) and 10q26 (open bars) and potential subtelomeric exchanges are depicted. Below, normal and hybrid and long or short *Eco*RI fragments derived from chromosome 4q35 or 10q26, are shown. Since the origin of the telomeric regions of the hybrid

fragments might be either chromosome 4q35 or 10q26, they are indicated by grey bars. Note that short, 4q35-linked, hybrid *Eco*RI fragments containing *Bln*I-sensitive repeated units are also diagnostic for FSHD1, but might lead to misdiagnosis when based on differential restriction analysis only.

7.6 Repeat array configurations

In a subsequent study, we examined the subtelomeric repeat array configurations on chromosomes 4q35 and 10q26 in 208 unrelated healthy individuals: 128 males and 80 females. Alleles were again sized by PFGE and assigned to their chromosomalorigin based on their *Bln*I sensitivity. Most of the individuals displayed a standard pattern of 4-type arrays on chromosome 4 and 10-type arrays on chromosome 10 (van Overveld *et al.,* 2000).

In accordance with the initial study, 21% of individuals displayed a non-standard repeat array configuration on chromosomes 4 and 10. 10-type repeat arrays on chromosome 4 were identified in 18 individuals (9%), whereas the reverse configuration (i.e. 4-type repeat arrays on chromosome 10) was present in 25 individuals (12%). One individual had such a complex repeat array configuration that it could not be equivocally assigned. Comparable frequencies have been seen in populations of other ethnic origins (Matsumura *et al.,* 2002; Wang *et al.,* 2003).

Studying all alleles in individuals carrying translocated repeat arrays, 10-type arrays on chromosome 4 tended to be more heterogeneous than 4-type arrays on chromosome 10. Of the translocated 10-type arrays on chromosome 4, 17% (6/36) were composed of a homogeneous array of 10-type repeat units whereas 33% (12/36) were hybrid arrays, consisting of clusters of 4-type and 10-type repeat units. Conversely, 44% (22/50) of chromosomes 10 carried a homogeneous translocated array of 4-type units only, whereas 10% (5/50) were hybrid arrays (van Overveld *et al.,* 2000).

7.7 Functional consequences

At present we can only speculate as to the functional consequences and evolutionary aspects of these exchanges. Van der Maarel *et al.* (2000) observed frequent somatic mosaicism in *de novo* FSHD families. Moreover, the challenging observation was made that the presence of a supernumerary 4-type repeat on chromosome 10 significantly increased the probability of mosaicism. The subtelomeric regions of chromosomes 4 and 10 tend to exhibit increased somatic pairing in interphase nuclei as studied by FISH (Stout *et al.,* 1999). All these observations suggest that there is cross-talk between the two regions. These interactions may play a role not only in the cause (rearrangement) but also in the mechanism underlying FSHD (position effect, etc.).

7.8 Repeat size distribution

To study the repeat-size distribution on both chromosomes, only alleles of individuals with a standard pattern were included in the analysis of allele size distribution *(Figure 7.4)* since this class represents the normal unbiased allele size distribution, whereas in other classes with heterogeneous repeat arrays, the allele sizes may be influenced by the translocated repeat arrays (van Overveld *et al.*, 2000). All alleles were grouped into intervals of 20 kb each.

An overall analysis showed a median of 4-type repeat arrays of 96 kb, whereas the median of 10-type repeat arrays was 75 kb. Whether this size difference reflects a biological or a mechanistic phenomenon remains to be elucidated. Interestingly, the repeat sizes on both chromosomes do not display a uniform distribution. The observed distributions for chromosomes 4 and 10 both agreed significantly better with a mixture of three normal distributions with equidistant means than with a mixture of two normal distributions. The best fit model therefore indicates that the allele sizes follow distributions with periodicity (van Overveld *et al.*, 2000).

The multimodal allele size distribution on both chromosomes with three equidistant peaks at intervals of 65 kb is intriguing. For minisatellite repeats, bimodal and trimodal distributions have been observed (Stead and Jeffreys, 2000). This is

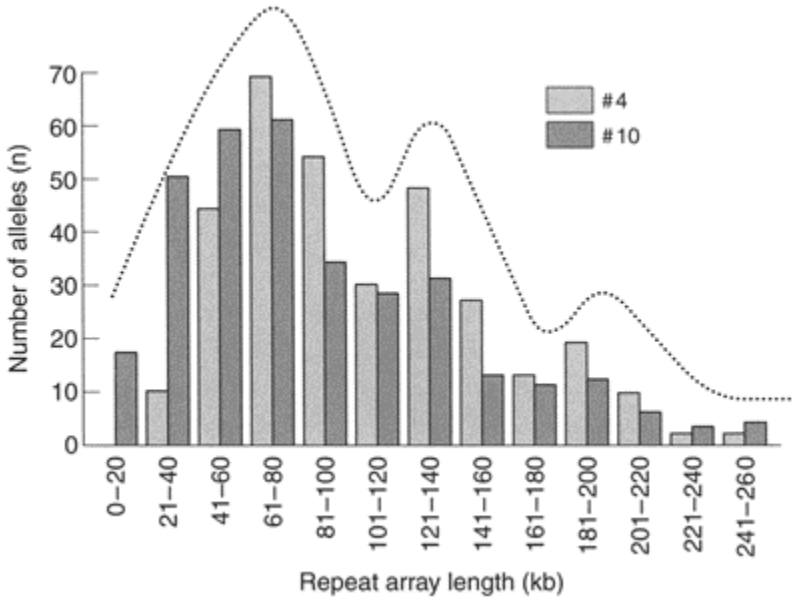

Figure 7.4 Repeat array size distribution of 4-type and 10-type repeat arrays from disomic individuals. On the *x*-axis, the repeat array length is plotted in intervals of 20 kb. The *y*-axis

displays the number of alleles in each size interval. Chromosome 4-type repeat arrays are represented by light bars, whereas 10-type repeats are denoted by dark bars.

mostly attributed to a founder effect of two ancestral alleles that have a similar dynamic behaviour with no evidence of interchromosomal interactions. Since we observed the same unequal size distribution on both chromosomes and have already demonstrated frequent interchromosomal cross-talk, a founder effect for these loci was highly unlikely. An alternative explanation, that the chromatin structure of repeat sequences may impose restrictions on repeat lengths, seems more likely. We had already demonstrated in mosaic individuals of *de novo* FSHD families that it is the shortest allele that usually rearranges to an FSHD-sized allele. This suggests that the unequal distribution on chromosome 4 reflects some kind of permutation state in the first peak as compared to the larger peaks.

7.9 FSHD-sized repeat arrays

Repeat arrays <38 kb and residing on chromosome 4 are associated with FSHD. In this survey, we identified 4-type repeat arrays of <38 kb in six individuals (van Overveld *et al.*, 2000). Except for one case, all these individuals carried a standard homogeneous repeat array configuration and consequently no apparent hints of exchange with chromosome 10. As the blood samples were obtained in a coded form from blood donors, no clinical information was available to further elucidate the FSHD status. Further studies to elucidate this finding are important for the discussion on the fragment size-dependent variation in penetrance or even the possibility of modifying genes. The recent finding by Lemmers *et al.* (2002) of a novel polymorphism in the subtelomere of 4q, with alleles 4qA and 4qB, is of importance. Only short fragments of the 4qA type were seen in FSHD patients. Remarkably, in the study presented above, the three shortest fragments were of the 4qB-type, while the longer fragments, borderline for FSHD, were of the 4qA-type (Lemmers *et al.*, 2002).

In conclusion, a detailed insight has been provided into the complex dynamic behaviour of the proximal subtelomeric domains on chromosomes 4 and 10 and may serve as a model for other subtelomeres. Although these domains share many properties and may frequently interact, they also display distinct differences in size and homogeneity. Their plasticity is emphasized by a very high somatic mutation frequency. This intriguing phenomenon is discussed further in Chapter 12.

Since the main difference between chromosomes 4q and 10q is the association between a short repeat array on chromosome 4 and FSHD, detailed studies have been initiated to understand the structure-function relationship. These two subtelomeres are becoming a playground for a growing number of scientists interested in evolution, recombination, chromatin structure, long-range gene regulation, etc. These complementary efforts will sooner or later lead us to the key of the FSHD enigma.

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8.
Genomic analysis of the subtelomeric regions of human chromosomes 10q and 4q: relevance to FSHD

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

8.1 Introduction

Telomeres define the most distal genetic and physical boundaries of chromosomes and consist of essential structures that ensure the stability of the genome. The human telomere itself consists of a distinct tandemly repeated structure of $(TTAGGG)_n$. Between this array and the unique chromosome-specific sequence, there is a complex and variable region of the chromosome: the subtelomere. This region varies between chromosomes, although there is extensive sharing of sequence domains suggesting a complex pattern of duplications between subtelomeres of different chromosomes (Flint *et al.*, 1997a, 1997b). Some of these duplication events are estimated to have occurred quite recently, between 5 and 25 million years ago (Martin *et al.*, 2002). The complexity and dynamic nature of these genomic regions is reviewed by Mefford and Trask (2002). In this chapter, we provide an overview of what is known about the organization of the human chromosome 4q and 10q telomeres. The relationships between these two subtelomeres and the implications for the FSHD disease mechanism are highlighted.

8.2 Genomic analysis of the FSHD locus on chromosome 4qter

The FSHD1 locus is located close to the telomere of chromosome 4q (Bengtsson *et al.*, 1994). Perhaps as a consequence of this terminal position, the genomic organization and relationship to other subtelomeric regions is quite complex. Initial physical mapping studies, such as Southern blot hybridization, cDNA selection and FISH, suggested that sequences closely related to loci on distal 4q35 (including but not restricted to the D4Z4 repeat) are dispersed throughout the human genome (Hewitt *et al.*, 1994; Winokur *et al.*,

1994; Altherr *et al.,* 1995; Lyle *et al.,* 1995; van Deutekom *et al.,* 1995). Using chromosome 4q35 probes for hybridization of human genomic libraries identified nine to ten times more positive PAC clones than expected, a finding consistent with the presence of many homologous regions in the genome (van Geel *et al.,* 1999). These duplicated regions (duplicons) are almost exclusively associated with telomeric and centromeric chromosomal regions, in particular those of the acrocentric chromosomes (van Geel *et al.,* 1999; Ballarati *et al.,* 2002).

All the coding or potentially coding sequence elements identified in the FSHD region (*FRG1, FRG2, TUBB4Q* and D4Z4) have been involved in these duplication events and therefore have homologous family members elsewhere in the genome (Hewitt *et al.,* 1994; van Deutekom *et al.,* 1996b; Ding *et al.,* 1998; van Geel *et al.,* 1999, 2000, 2002b). The complex duplicon arrangement of chromosome 4q35 has complicated both the genomic analysis of this region and the molecular diagnostics of FSHD.

To characterize the region of chromosome 4q35 distal to D4Z4, we used a combination of YAC, BAC, PAC, phage and cosmid clones (van Geel *et al.,* 2002a). Examination of clones and DNA sequence showed that a long (around 8 kb) array of 68 bp satellite DNA is juxtaposed to D4Z4, followed by a region containing many pseudogenes and low copy repeats. Since none of the clones used in the initial analysis extended to the telomere itself, a half-YAC clone (RM2173) was then used. Half-YACs (which as the name suggests contain a single telomere in the vector and rely upon complementation by a human telomere array in the cloned DNA) are an extremely useful resource for generating physical and sequence maps of human telomeres (Riethman *et al.,* 2001).

8.2.1 A polymorphism at the 4q telomere

Mapping data from YAC RM2173 suggested that it might contain a variant of 4qter (termed 4qB) and this was confirmed by DNA sequencing. Although both the 4qA and 4qB sequences were produced from YAC clones originating from human chromosome 4, distal to D4Z4 they have only 92% nucleotide identity. The organization of sequence elements within 4qA and 4qB showed significant variations *(Figure 8.1)*. Within 4qA there is an 8 kb region of 68 bp satellite DNA immediately distal to the D4Z4 and adjacent to this is a 1 kb divergent $(TTAGGG)_n$ array. Neither of these repeat arrays is present within the 4qB sequence. The location of the junction between the D4Z4 repeat array and the adjacent subtelomeric sequence is also different between the two alleles. In 4qB the terminal 3.3 kb repeat contains only the first 570 bp of a complete unit, whereas in 4qA the terminal repeat is a divergent 3.3 kb repeat (pLAM). Hybridization of human genomic hybridization data with a 4qB-specific probe (just distal of the 3.3 kb array) confirmed the presence of the two different alleles in the Dutch population (Lemmers *et al.,* 2002). This polymorphism is discussed further in Section 8.6.

8.3 Extensive sequence homology between chromosome 4qter and 10qter

Database searching shows that the distal 10–12 kb of the 4q telomere has sequence similarity to many chromosomes, including 21q and 22q *(Figure 8.1)*. However, the most extensive sequence similarity is between chromosomes 4qter and 10qter. This

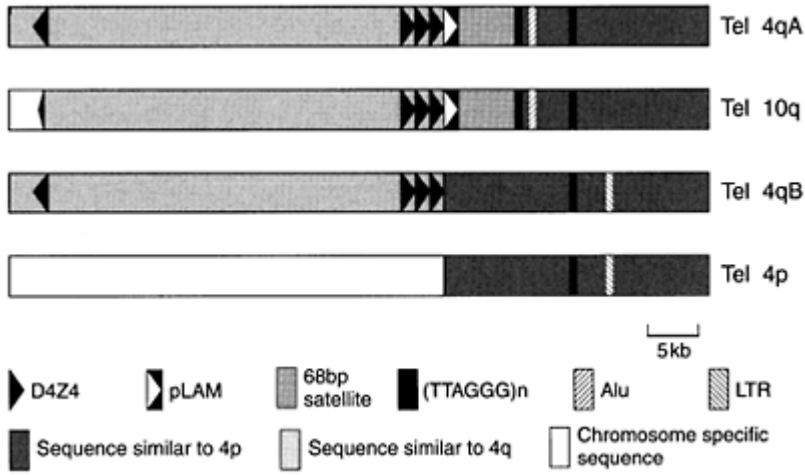

Figure 8.1 Schematic representation of 4qA, 4qB, 10q, and 4p subtelomeric regions. The sequence elements and regions of sequence similarity are indicated by shading (see keys). The proximal homology boundary between 4q and 10q occurs within an inverted, upstream D4Z4 repeat, with only approximately half the repeat unit being retained on chromosome 10.

will be discussed with respect to three regions; D4Z4, the subtelomere, and the region proximal to the 3.3 kb repeats.

8.3.1 The 4q35 and 10q26 3.3 kb repeats

With the identification of p13E-11 as a probe for detection of the D4Z4 deletion associated with FSHD, a tool for diagnostic DNA analysis for genetic counselling became available (Bakker *et al.*, 1996). However, such analysis has been complicated by

cross-hybridization of p13E-1 1 to another polymorphic locus on chromosome 10q26 (Bakker *et al.,* 1995; Deidda *et al.,* 1995). Although there is evidence for genetic heterogeneity in FSHD, no families show linkage to chromosome 10q26 (Bakker *et al.,* 1995; Gilbert *et al.,* 1995). Detailed analysis of the 10q26 locus indicates a similar arrangement of 3.3 kb tandemly repeated units and flanking sequences (Deidda *et al.,* 1995). As with the 4q35-linked p13E-11 *Eco*RI DNA fragments, the polymorphic 10q26 fragments range in size from approximately 10 to >300 kb (Wijmenga *et al.,* 1994). However, small (<35 kb) fragments on chromosome 10 are *not* associated with FSHD or any other disease.

Therefore, chromosome 10q26-associated p13E-11 *Eco*RI fragments of less than 35 kb, which occur in at least 10% of the human population, may incorrectly be assigned to 4q35 deletion fragments, leading to possible FSHD misdiagnosis (Bakker *et al.,* 1996). The reliability of molecular diagnosis with probe p13E-1 1 was significantly improved by identification of a unique *Bln*I restriction site only present in 10q26 3.3kb repeated units (Deidda *et al.,* 1995, 1996). Southern blot analysis on *Eco*RI-*Bln*I doubly digested genomic DNA enables direct differentiation of short 4q35 fragments, since 10q26-specific *Eco*RI segments are eliminated by *Bln*I digestion. More recently, it has been shown that *Xap*I differentially digests the 4q35 repeats (Lemmers *et al.,* 2001).

In the majority of FSHD cases, the use of a double digest in combination with Southern blotting results in a reliable diagnosis. However, a small yet significant number of chromosomes 4 carry 10-type 3.3 kb repeats (as defined by the presence or absence of *Bln*I or *Xap*I sites) or *vice versa*. In such cases, Southern blot data indicate the presence of only one or three chromosome 4q35 fragments (van Deutekom *et al.,* 1996a). Of course, there are still two chromosome 4 alleles and two chromosome 10 alleles, but it is not possible to assign fragments unambiguously. The existence of chromosome 4q35-specific 3.3 kb repeat arrays on chromosome 10q26 and *vice versa* is apparently relatively common, with at least 20% of individuals in Caucasian populations having such haplotypes (van Deutekom *et al.,* 1996a; Overveld *et al.,* 2000). To further complicate analysis, D4Z4 arrays may have a hybrid structure composed of a combination of BlnI-sensitive and -resistant repeat units (Lemmers *et al.,* 1998).

However, FSHD1A is *only* associated with a short 3.3 kb repeat array on chromosome 4q35, regardless of the repeat composition. Hence, it is the number of D4Z4 repeats that is critical and not their sequence. This suggests that the repeated units probably do not contain the FSHD gene, and therefore supports the position effect hypothesis as the most likely mechanism for FSHD. In addition, healthy individuals have been described with deletions proximal to the D4Z4 locus, encompassing p13E-11 but not the repeats themselves (Lemmers *et al.,* 1998). Delimiting the boundaries of these deletions could be instrumental in refining the critical region for FSHD.

8.3.2 Sequence homology between the 4q and 10q subtelomeres

It is unclear why there is no association between short alleles at the 10q repeat locus and FSHD or, as far as is known, any other disease phenotype. Assuming the position effect mechanism is correct, it would be expected that deletion within the 10q26 repeat array would also induce changes in chromatin structure. One possible reason for the lack of an effect is that the telomeres of the two chromosomes have very different arrangements.

Therefore, we investigated the organization of both telomeres by YAC and PAC analysis of the terminal regions of both chromosome ends.

Clones containing the subtelomeric region from chromosome 10q were isolated from a genomic human PAC library (RPCI-6) by hybridization with p 13E-11. A combination of STS-PCR screening and restriction enzyme fingerprinting was used to determine the chromosomal origin of the PAC clones. The 3.3 kb repeats from the 10q-specific PAC clones were further analysed by *Eco*RI/*Bln*I restriction mapping and a clone containing six 3.3 kb repeats (RPCI-6-112J1) was selected for sequencing (van Geel *et al.*, 2002a). The distal end of PAC RPCI-6-112J1 maps within the 68 bp satellite DNA array on 10q. PCR and sample sequencing were therefore used to analyse the more distal subtelomeric region using a chromosome 10 half-YAC (RM2136).

The data suggest that the 10q subtelomeric region distal of the 3.3 kb repeats has a strikingly similar organization to the 4qA subtelomere (van Geel *et al.*, 2002a). Since this work was published, the sequence of a 37 kb cosmid cloned from RM2136 has been deposited in GenBank (Accession Number AL732375). Examination of this sequence confirms our findings, with a 68 bp satellite DNA immediately distal of the 10q 3.3 kb repeats and approximately 98% nucleotide identity between the 4qA and 10ter subtelomeres. However, for both chromosomes, this subtelomeric sequence is not yet contiguous with the telomeric array. What is of interest is that with respect to both overall nucleotide identity and arrangement of sequence elements *(Figure 8.1)*, the 10q subtelomere is more similar to 4qA than the two 4q alleles are to each other. This indicates that 4qA and 10qter arose via a recent duplication of a common progenitor.

8.3.3 Sequence homology between chromosomes 4q35 and 10q26 extends proximal to the 3.3 kb repeats

Large-scale sequence analysis was performed on the region proximal to the 10q26 3.3 kb repeats to define the proximal homology boundary and to search for genes on chromosome 10 that might be influenced by a position effect (van Geel *et al.*, 2002a). Comparison of the 4q and 10q sequences shows that the homology extends 42 kb proximal to D4Z4 *(Figure 8.1)*. *FRG2* is located within this region of homology.

In the sequence uniquely associated with chromosome 10q26, a gene was identified approximately 96 kb proximal to the 3.3 kb repeats (van Geel *et al.*, 2002a). The mRNA is 1.3 kb in length and encodes a putative protein of 282 amino acids. The expression of the mRNA was restricted. RT-PCR amplification was only noted in testis and hypothalamus. Searches with the protein sequence in the peptide database resulted in no significant hits and modular domain pattern homology was unable to classify this putative protein. So far, no assessment of gene expression levels in relation to 3.3 kb repeat copy number on chromosome 10q26 has been carried out.

8.4 Relationship of 4qter to other telomeres

BLAST searching with 4qA subtelomeric sequence (GenBank Accession Number AF017466) yields significant hits with many genomic regions, notably 4pter (Flint *et al.*, 1997b), but also 21qter, 22qter, 18pter and 1qter, and several unassigned clones. The full

extent of the relationships between different subtelomeric regions awaits the completion of sequencing of all the telomeric regions and integration of these data with the human genome sequence (Riethman *et al.*, 2001). Progress in the human telomere project may be monitored at www.wistar.upenn.edu/Riethman/.

8.5 Evolution of 4qter

The homology boundaries between 4pter and 4qA are located at the 68 bp satellite/D4Z4 repeats *(Figure 8.1)*. The nucleotide identity between the 4qA and 4qB alleles is only 92% *(Figure 8.1)*. The 4qA allele most closely resembles the 10q26 subtelomere, while the 4qB allele has higher similarity to 4p (>99% nucleotide identity). An evolutionary model is proposed for the dynamic 4q telomere, which may have been involved in shaping the contemporary telomeres of 10q, 4p and probably many other chromosomal subtelomeres *(Figure 8.2)*. Initially, there was a duplication event involving the 4p and 4q telomeres. However, the ancestral sequence could have been present on 4p or 4q, or even a different chromosome. A second, perhaps concurrent event then duplicated at least part of this region onto additional chromosomes.

Following the duplication of 4p/4q subtelomeric sequence, the two subtelomeric regions diverged and acquired different sequence elements. For example, we identified an *Alu* insertion into the 4q subtelomere and a LTR insertion into 4p (van Geel *et al.*, 2002a). The distal end of 4q, including the 3.3 kb repeats, was then duplicated onto chromosome 10q. Evidence for chromosome 4qter being the progenitor sequence has come from studies of the evolution of D4Z4 in primates. In great apes and Old World monkeys, this repeat maps to loci homologous to chromosome 4qter (Clark *et al.*, 1996; Winokur *et al.*, 1996). The 10q and 4qA regions are likely to have originated from a common ancestral sequence since they both contain the 3.3 kb repeats and the 4q *Alu* repeat, whilst the 4p LTR repeat is absent. Finally, the 4qB allele was produced more recently by a further duplication of the 4p subtelomeric sequence onto 4q, perhaps through misalignment of the two telomeres and recombination within the subtelomeric domains. It has been suggested that subtelomeric domains are swapped by recombination-mediated exchange occurring between different chromosomes at internal telomeric arrays (Chute *et al.*, 1997; Flint *et al.*, 1997a, 1997b; Mefford and Trask, 2002). However, although such arrays are present within 4q and 10q, they do not define the duplication boundaries *(Figure 8.1)*.

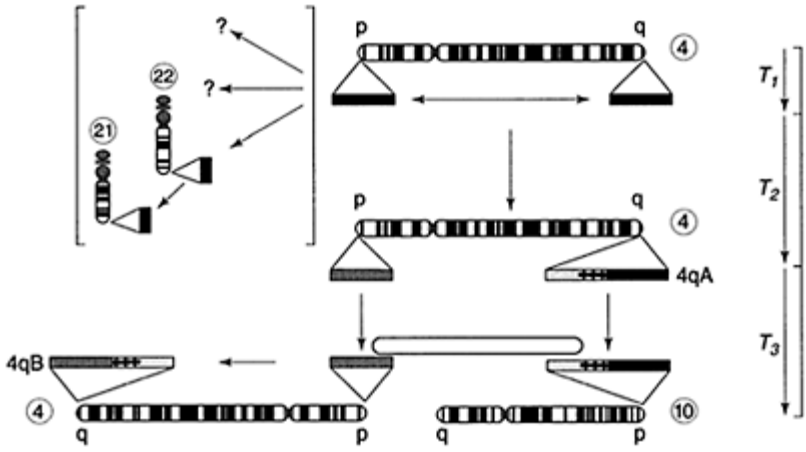

Figure 8.2 Proposed mechanism for evolution of the 4q telomere. The first step of this model is the duplication event involving the 4p and 4q telomeres at time T1. The origin of this telomere may have been another chromosome. This region was also duplicated on to additional chromosomes, including 21q and 22q, the timing of this event is uncertain. Following these duplication events, 4p and 4q diverged by the insertion of an *Alu* repeat into 4q and an LTR insertion into 4p at time T2. The distal end of 4q was then duplicated on to chromosome 10q at time T3. In the final step of this model, the 4qB allele was produced by duplication of the 4p telomere onto 4q distal to the 3.3 kb repeats.

8.6 FSHD is only associated with the 4qA allele

Segmental polymorphisms at proterminal regions are found at particular subsets of human chromosomes and are frequently present within the human population (Der-Sarkissian *et al.*, 2002). We have shown that chromosome 4qter exhibits similar

properties. The 4qA and 4qB polymorphic alleles on chromosome 4 are found at almost equal frequencies in the healthy population. One of the most important papers to come out of the FSHD field in recent years has come from a study by the Leiden group which demonstrated that FSHD deletions in D4Z4 are associated only with the 4qA allele (Lemmers *et al.*, 2002).

What mechanistic processes underlie this preferential association of the 4qA allele with FSHD are presently unknown. One hypothesis is that 4qA alleles are more recombinogenic than 4qB alleles. However, six unaffected individuals have been described with non-pathogenic *de novo* contractions, three of which involve 4qB alleles (Lemmers *et al.*, 2002). Therefore, the most likely hypothesis is that there is a functional difference between the two alleles that results in deletions only being associated with 4qA being pathogenic.

Recently, a model was proposed that D4Z4 deletions result in transcriptional derepression of 4q35 genes, leading to FSHD (Gabellini *et al.*, 2002). However, this model does not account for the strong 4qA allele association with FSHD. Some unique characteristic of 4qA must cause or facilitate the transcriptional derepression, or, by contrast, the 4qB allele must protect against it. The most prominent difference between the two alleles is the presence of the 68 bp (β-satellite) repeat. These repeats are often associated with heterochromatin and may therefore be intrinsic to a D4Z4-deletion-mediated chromatin alteration resulting in transcriptional gene derepression and FSHD. Specific protein-binding sites for 68 bp satellite repeats may be involved in this process. Once again though, a simple answer cannot account for all the data; since the chromosome 10q subtelomere is essentially identical to the 4qA allele including the 68 bp satellite repeats, similar transcriptional derepression processes would also be expected to occur at this locus. Therefore, such a mechanism must accommodate the fact that transcriptional alteration from 10qter genes by similar D4Z4-contraction events fails to impact critically upon the metabolic stability of the cell and to result in disease.

8.7 Concluding remarks

We have shown that the 4q and 10q subtelomeric regions share extensive sequence homology, suggesting that the apparent lack of pathogenic effect of deletions within the 10q 3.3 kb repeats is not due to a different telomere organization. A polymorphic variant of the 4q telomere has also been identified. Recently, FSHD has been shown to be solely associated with only one of these variants, the 4qA allele. Further studies on the functional differences between the two forms of the 4q telomere will now be important and are likely to provide important insights into the FSHD disease mechanism.

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9.
The DUX gene family and FSHD

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9.1 Introduction

Classical genes only constitute a small part of the human nuclear genome alongside numerous repetitive elements of various sizes and copy numbers. Some of these are clustered in tandem repeat arrays, amplified by unequal crossover or unequal sister chromatid exchanges. Some constitute families of homologous sequences dispersed in the genome, are remnants of transposable elements that have integrated into various locations by mechanisms involving occasionally a DNA or more frequently an RNA intermediate. A few of these still encode the enzymes needed for their transposition, and a number of human mutations have been found to be caused by such insertional events (e.g. Sukarova *et al.,* 2001). A cellular defense mechanism to prevent damage by active transposition is the methylation of CpG sequences in repeated elements (Walsh *et al.,* 1998). This small chemical modification will contribute to heterochromatin assembly, a compact protein/DNA structure that inhibits transcription, recombination and transposition. A first step is the recruitment of methyl CpG-binding proteins that associate with histone deacetylases (HDACs). These enzymes target the acetylated lysines of the nucleosomal histones, restore their positive charges thereby increasing their ionic interactions with the phosphate groups of DNA. The resulting compacted nucleosomes will recruit histone H1 and non-histone proteins such as HP1 to further condense into heterochromatin (e.g. Saveliev *et al.,* 2003).

The chromatin structure is erased at the time of cell division when DNA replication occurs, and is usually rapidly reconstituted. In yeast, it was found that about every 10–20 generations a heterochromatic region could open and allow weak expression of a previously silenced gene (reviewed in Ptashne and Gann 2002). Sometimes the repeat-induced heterochromatin extends outside of the repeated elements and affects expression of neighbouring genes. A change in the extent of heterochromatization after DNA

replication might thus affect expression of such genes, a phenomenon initially observed in *Drosophila* and named position effect variegation (PEV; reviewed in Weiler and Wakimoto 1995). Repeat-induced silencing has also been observed in transgenic mice obtained by zygote injection. Indeed, the injected transgene molecules first form a concatemer before integrating into a mouse chromosome. A high transgene copy number increases the concatemer length and the probability of heterochromatization, precluding expression. This was demonstrated in mice where different copy numbers of a *lacZ* transgene were integrated in an identical chromosome site (Garrick *et al.,* 1998).

9.2 Cloning of a gene promoter homologous to part of the D4Z4 unit

Within the context of another project, our group has characterized a transcription factor that could activate basal expression of the plasminogen activator inhibitor-1 *(PAI-1)* gene in synergy with the ubiquitous transcription factors Sp1 and Sp3 (Ding *et al.,* 1996, 1999). This protein presented the seven conserved domains of a DNA helicase, and was named Helicase-like Transcription Factor (HLTF; SMARCA3 in OMIM). Its structure is similar to members of the SWI/SNF family of transcriptional regulators that act by remodeling chromatin structure (e.g. Boyer *et al.,* 2000). Since HLTF possessed a unique DNA-binding domain absent in other SWI/SNF family members, we wanted to identify its target genes by a chromatin immunoprecipitation approach. Nuclei were prepared from HeLa cells known to express large amounts of HLTF, and digested by a restriction enzyme to generate DNA fragments retaining the proteins that they were associated with in chromatin. The HLTF-bound DNA fragments were immunoprecipitated with a monoclonal antibody directed against HLTF and previously shown to stabilize HLTF/DNA complexes *in vitro,* in electrophoretic mobility shift assays (EMSA). The immunoprecipitated fragments were cloned in a bacterial plasmid, their capacity to bind HLTF was checked in EMSA and their sequences determined. Among these, a 182 basepair (bp) fragment containing a putative TATAA box was selected for further studies, and was named *HEFT1* for *Helicase-like transcription factor target 1* (Ding *et al.,* 1998).

A search for homologous sequences in the GenBank/EMBL databases with the BLAST algorithm (Altschul *et al.,* 1997) pointed to a repeated segment of the 4q35 locus (D4Z4) that had been sequenced by Jane Hewitt's group from a human genomic DNA library (Hewitt *et al.,* 1994; GenBank Accession Number # L32607). This polymorphic locus had previously been linked to FSHD: non-affected individuals present 11–100 tandem copies of the D4Z4 repeated element whilst one chromosome 4 allele of patients has undergone a deletion leaving only one to ten repeats (Wijmenga *et al.,* 1992). The D4Z4 unit had been defined as a 3.3 kb *Kpn*I element comprising several GC-rich repeat elements usually associated with heterochromatin, and a large open reading frame (ORF) across two homeoboxes. However, since no corresponding promoter nor transcript had been identified, it had been concluded that no functional gene was present in D4Z4. Our *HEFT1* promoter presented 87% nucleotide identity with a region upstream of the two homeoboxes in D4Z4, but, intriguingly, this sequence was *within* the previously defined ORF *(Figure 9.1)*. The *HEFT1* putative TATAA box corresponded precisely with a TACAA sequence in D4Z4, a mutation known to diminish transcription about

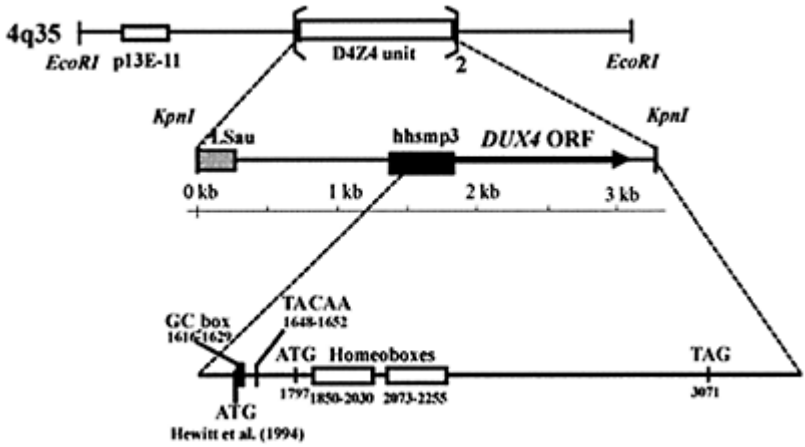

Figure 9.1 Schematic representation of the FSHD locus. Top: *Eco*RI fragment comprising the 4q35 locus of a patient with only 2 D424 units left, and the *p13E11* probe used for genotyping. Middle: enlargement of the D4Z4 unit as a 3.3 kb *Kpn*I fragment with the *LSau* and *hhspm3* repeat regions and the *DUX4* open reading frame (ORF). Bottom: the *DUX4* gene with the GC and TACAA boxes downstream from the start codon of the large ORF (ATG; Hewitt *et al.*, 1994) but upstream from the shorter *DUX4* ORF (Gabriëls *et al.*, 1999).

fivefold in another system, but not to suppress it (Wobbe and Struhl, 1990). A shorter ORF could be identified, with a putative start codon located 135 bp downstream of the TACAA box, in frame with the two homeoboxes (Ding *et al.*, 1998). This observation raised the provocative hypothesis that a putative homeotic gene might be present within the D4Z4 unit.

9.3 Expression of double homeobox *(DUX)* genes located within 3.3 kb elements not linked to FSHD

Members of the 3.3 kb repeat family were previously detected by fluorescent *in situ* hybridization using the double homeobox as a probe on the short arms of all the

acrocentric chromosomes where they were partially interspersed with the ribosomal RNA gene clusters (Lyle *et al.,* 1995; Winokur *et al.,* 1996). Indeed, several attempts at precisely mapping *HEFT1* by amplification of sequence tagged sites (STS) on a panel of rodent/human hybrid cells each comprising a different human chromosome yielded the same signal on all the acrocentric chromosomes. The frequent exchanges that occur between these short arms explain why very similar repeated sequences can be found on every acrocentric chromosome (Ding *et al.,* 1998; Beckers *et al.,* 2001).

Owing to its location in repeated elements considered as 'junk DNA', the questions to be addressed were whether the *HEFT1* putative promoter was active, and whether it was part of a functional gene. When fused to a luciferase reporter gene, *HEFT1* displayed promoter activity in transfected HeLa or human rhabdomyosarcoma TE671 cells. The origin of transcription was mapped to a nucleotide located 25 bp downstream of the TATAA box. Further transient expression studies on *HEFT1* mutants showed that basal expression was mediated by a GC box located 30 bp upstream of the TATAA box, and activated by interaction with the ubiquitous Sp1 or Sp3 transcription factors (Ding *et al.,* 1998).

A search for a cDNA homologous to *HEFT1* was based on the assumption that it was part of a 3.3 kb element similar to the published D4Z4 sequence. Reverse transcription (RT) was performed on TE671 total RNA with a 3′ primer mapping downstream of the D4Z4 homeoboxes. This cDNA strand was amplified by PCR with the addition of a 5′ primer derived from the short *HEFT1* transcribed region. A 1.2 kb RT-PCR product was cloned and sequenced, and showed 88% nucleotide identity to D4Z4. It contained a 170-codon open reading frame (ORF) across the homeoboxes, and was named *DUX1* for double homeobox 1. This protein was produced *in vitro* by transcription/translation of the *DUX1* ORF (Ding *et al.,* 1998).

Although the *HEFT1* promoter and the *DUX1* cDNA presented a single mismatch in their overlapping region, they were not derived from the same gene. The missing part of each gene had to be sought by a strategy that allowed for the fact that hundreds of 3.3 kb elements with homologous *DUX* genes are dispersed in the human genome (Lyle *et al.,* 1995; Clark *et al.,* 1996; Winokur *et al.,* 1996). Primers 'specific' for a given family member were designed on the basis of an alignment of the few known *DUX* sequences and used for PCR on genomic DNA. The amplification products were mixtures of fragments derived from different *DUX* genes: they were inserted in a bacterial plasmid, and separated by cloning to allow determination of the individual sequences. These were included in the alignments, and new, more specific, primers were selected to repeat the process. The *DUX2* gene was cloned in this way: it encompassed the *HEFT1* promoter and a *DUX1*-like ORF with a stop codon between the homeoboxes (Ding *et al.,* 1998). In order to decrease the family complexity, a similar strategy was used on phage P1-based artificial chromosomes (PACs) selected from a human genomic library for the presence of *DUX1*-like sequences. A *DUX3* gene was cloned that encoded a protein presenting four mismatches with DUX1. PCR on human genomic DNA with the same primer combination yielded the 98% similar *DUX5* gene that encoded a protein identical to DUX1. Both genes also encoded a 27-residue longer protein differing by alternative translation initiation in the same ORF (Beckers *et al.,* 2001).

As a way to evaluate expression of these genes, RT-PCR was performed on total RNA from several human tissues (liver, muscle, kidney) with a 5′ primer coupled to the T7

RNA polymerase promoter: the amplified products were submitted to transcription/translation *in vitro* and yielded two proteins of 22 and 19 kDa, sizes expected for the *DUX3* and *DUX5* gene products. By contrast, no RT-PCR product could be obtained from poly(A)$^+$ RNA (Beckers *et al.,* 2001).

Taken together, these data suggest that a number of 3.3 kb elements comprise functional *DUX* genes that have not been detected because they map to regions excluded from the human genome sequencing project. Moreover, their mRNA sequences are not found in cDNA sequence (EST) databases because these were derived from poly(A)$^+$ RNAs.

9.4 Expression *in vivo* of a DUX1 protein with properties of a transcription factor

The 180 bp homeobox sequence is conserved in a number of genes encoding transcription factors involved in cell fate determination during embryo development. The corresponding 60 amino acid homeodomain consists of three α-helices with a precise spatial arrangement that allows specific DNA binding. The DUX1 homeodomains are homologous to the paired-type subfamily, with the highest similarity to PAX3 and PAX7, which are involved in muscle differentiation during embryogenesis and in regeneration of adult muscles (reviewed in Buckingham *et al.,* 2003). The ninth residue of the third α helix defines the spacing between the two halves of the TAAT/ATTA consensus binding site of the homodimer (Wilson *et al.,* 1993). In contrast to PAX3 and PAX7 where this residue is a serine, it is a glutamine in both DUX1 homeodomains suggesting a 5 bp spacing. Indeed, *in vitro* translated DUX1 specifically interacted in electrophoretic mobility shift assay (EMSA) with an oligonucleotide harboring such a P5 target. Moreover, transient expression of a minimal promoter-luciferase construct fused to a P5 oligonucleotide was induced up to fivefold by co-transfection with a DUX1 expression vector. Evidence for DUX1 expression *in vivo* was obtained by studies on human rhabdomyosarcoma TE671 cell extracts. First, a protein with identical migration as the *in vitro*-translated DUX1 was detected on a Western blot incubated with either a radioactive P5 oligonucleotide or a rabbit antiserum raised against a DUX1 peptide NH2 terminal to the homeodomains. Second, EMSA performed with the P5 oligonucleotide and TE671 cell extracts revealed a protein/DNA complex with identical migration as the *in vitro* translated DUX1/P5 complex. This complex was specifically suppressed by addition of the anti-DUX1 rabbit serum (Ding *et al.,* 1998).

9.5 A putative *DUX4* gene in the D4Z4 units of the FSHD locus

The above-mentioned experiments showed that some 3.3 kb elements contained *DUX* genes that were actively transcribed, and at least one of which was responsible for expressing a protein *in vivo* (DUX1). The sequence of the characterized *HEFT1* promoter corresponded to a putative promoter in the D4Z4 unit *(Figure 9.2A),* and the start codon of the *DUX1/2* ORFs precisely matched an ATG downstream of the TACAA box and in-frame with the D4Z4 homeoboxes. This suggested the presence of a *DUX4* gene in each

D4Z4 unit. This putative *DUX4* gene had initially been detected on control genomic DNA, and its presence was confirmed in a patient with FSHD, based on the sequence of the two D4Z4 copies left in his partially deleted 4q35 locus (Gabriëls *et al.*, 1999).

Doubt was cast on the functionality of the putative *DUX4* gene because it presented features of a pseudogene such as the mutation of its TATAA box to TACAA, and the lack of introns and polyA addition signal. We therefore first assessed the activity of its putative promoter. A 191 bp fragment homologous to the previously studied *HEFT1* promoter was linked to the luciferase reporter gene and evaluated in transient expression experiments, demonstrating a 25–30-fold higher activity in transfected human rhabdomyosarcoma TE671 cells as compared to non-muscle

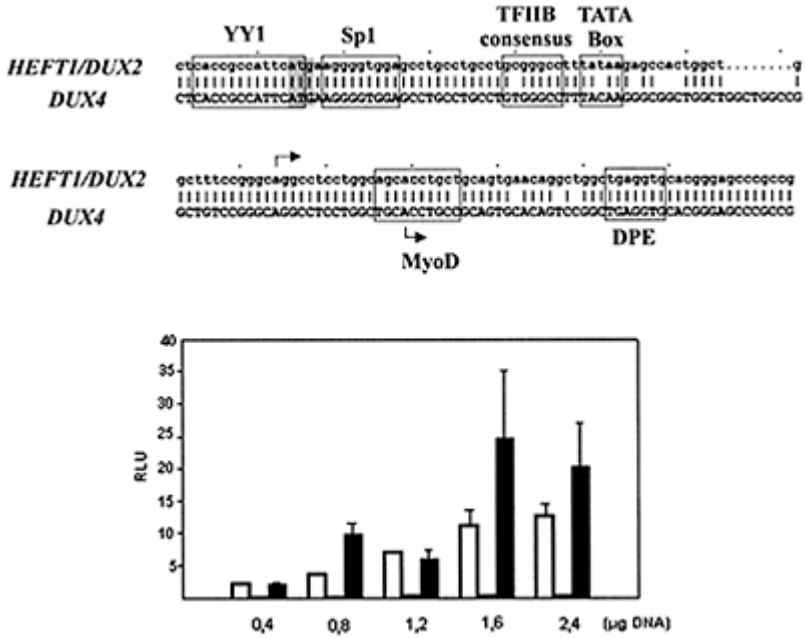

Figure 9.2 A. Sequence alignment of the *HEFT1* and *DUX4* promoters. The boxed *cis*-elements are from 5′ to 3′: the YY1 binding site, the GC box (Sp1 binding site), the TFIIB consensus binding site, the TATAA box, a putative MyoD binding site, and a consensus downstream promoter element. The arrows indicate the experimentally determined transcription start sites. The

highlighted ATG is the ORF start codon initially proposed by Hewitt *et al.* (1994). B. Transient expression analysis of the *DUX4-LUC* activity upon YY1 site mutation. 4×10^4 C2C12 cells were plated in each well of a 6-well culture dish and grown in DMEM with 10% fetal calf serum. Cells were transfected 24 h later with Lipofectamine 2000 (Invitrogen) and the indicated amounts of the pGL3 (Promega) luciferase vector containing the *DUX4* promoter either wild-type (*pGL3-DUX4;* white bars), mutated in the GC box (*pGL3-DUX4GCmut;* grey bars), or in the YY1 site (*pGL3-DUX4YY1mut;* black bars). The culture medium was removed after 20 h, and cells of individual wells were lysed in Bright Glow buffer (Promega) followed by the luciferase assay. The data are given as relative light units and normalized to the amount of proteins (Bradford assay) in each sample (duplicates).

HeLa cells. Mutations in the TTACAA box known to increase (TTTAAA) or decrease (TTCCAA) its efficiency affected *DUX4-LUC* transient expression as expected. The GC box mutations (GGAATGG) known to strongly affect the homologous *HEFT1* promoter activity reduced *DUX4-LUC* expression tenfold in TE671 cells (Gabriëls *et al.*, 1999) and 30-fold in C2C12 mouse myoblasts *(Figure 9.2B).*

A *cis*-element has recently been identified in the D4Z4 unit that, upon multimerization, repressed a linked reporter gene in HeLa cells (Gabellini *et al.,* 2002). The protein complex mediating this inhibition was characterized, and its DNA-binding component identified as YY1 (yin yang protein 1), a *trans*-factor known to activate or repress transcription according to the cell type and promoter context (e.g. Bauknecht *et al.,* 1996; Galvin and Shi, 1997). Gabellini *et al.* (2002) described a mutation (CCGCCGCCCAT) that prevented YY1 binding in EMSA, and relieved repression in transfected HeLa cells. This repressing *cis*-element precisely mapped next to the GC box of the homologous *HEFT1* and *DUX4* promoters *(Figure 9.2A).* We evaluated its role by introducing this inactivating mutation in the *DUX4-LUC* construct. If indeed the YY1

cis-element was inhibitory in muscle cells, one would expect this mutation to activate the *DUX4* promoter. Transient expression experiments indicated that this construct had an activity in C2C12 cells that was not significantly different from that of the wild-type promoter *(Figure 9.2B)* shedding doubt upon its inhibitory function in muscle. In addition, high levels of YY1 in non-differentiated myoblasts are known to maintain several muscle-specific genes at a low transcription level. Differentiation to myotubes induces specific proteolytic degradation of YY1 by m-calpain and the 26S proteasome, allowing increased expression of these genes (Walowitz *et al.*, 1998). For instance, the dystrophin *(DMD)* gene promoter contains a YY1 site: it drives low expression of a linked luciferase gene in transfected C2C12 cells, and is induced fivefold upon differentiation to myotubes (Galvagni *et al.*, 1998).

9.6 Expression of the DUX4 protein

In order to test the hypothesis that the *DUX4* gene was involved in FSHD, its expression had to be evaluated in myoblasts or muscle biopsies from controls and patients. We first tried to detect *DUX4* RNAs by RT-PCR. However, we have not yet been able to define primers that would be specific to *DUX4* transcripts against the background of the highly similar RNAs expressed from *DUX* genes unlinked to FSHD. When RT-PCR was performed with a *DUX4* 'specific' primer pair on human muscle total RNA, we could isolate and sequence 17 individual *DUX*-like cDNAs. They constituted four highly similar groups, and a representative sequence of each is given in *Figure 9.3*. The multiple microheterogeneities in these sequences precluded the design of a primer pair more specific to *DUX4* and we could not use the recurrent strategy that succeeded for *DUX1, 3, 5* (see Section 9.3).

In the hope of reducing the *DUX* family complexity, we switched to protein detection, surmising that not every transcript would be translated. The putative *DUX4* gene includes an ORF starting 135 bp downstream from the TACAA box and encoding a 424-residue protein with two homeodomains. This protein is very similar to DUX1 except for a much longer carboxyl-terminal domain *(Figure 9.4)*. Consequently, the rabbit serum we developed against DUX1 reacted with DUX4 and other double homeodomain proteins. This serum could however be used for immunoblotting of two-dimensional gels that provide high resolution of complex protein mixtures (O'Farrel, 1975). In this technique, the proteins are first separated according to their charge by isoelectric focusing (IEF). They are separated in a second dimension according to their size by electrophoresis in the presence of sodium dodecyl sulfate in a polyacrylamide gel (PAGE-SDS). The protein extraction conditions were optimized with human TE671 rhabdomyosarcoma cells in order to solubilize the nuclear and low abundance DUX proteins and to ensure proper migration during IEF in the high pH range appropriate for the resolution of

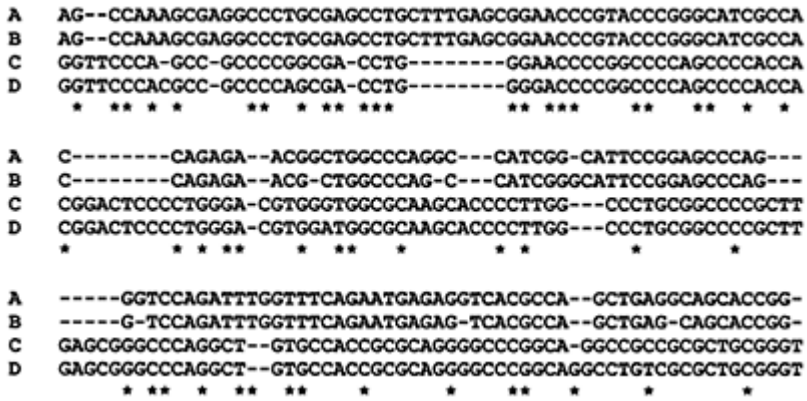

Figure 9.3 Heterogeneity of the *DUX* sequences co-amplified by RT-PCR with *DUX4*-specific primers. Human muscle total RNA (Stratagene) was reverse transcribed 30 min at 70°C by *Carboxydothermus hydrogenoformans* DNA polymerase (Expand High Fidelity kit, Roche) in the presence of 5% DMSO with a primer designed on the *DUX4* gene sequence 3′ from the second homeobox (5′ TCG CCA GGA GCT CAT CCA GCA GCA G 3′; antisense). Amplification was performed after denaturation for 4 min at 94°C, with High Fidelity *Taq* DNA polymerase (Roche) in the presence of 2 M betain for 35 cycles (94°C, 1 min; 66°C, 1 min; 72°C, 1 min 30 s) with the same 3′ primer and a 5′ primer upstream from the first DUX4 homeobox (5′ GCA CCT GCC GCA GTG CAC AGT CCG 3′; sense). The amplified fragments were cloned in the *pCR4TOPO* vector (Invitrogen) after incorporation of a protruding A by incubation for 15 min at 72°C with *Taq* DNA polymerase (MBI

Fermentas). After transformation of *E. coli*, 17 individual clones were selected, and plasmid DNA extracted for sequence determination (CEQ DTCS-Dye terminator Cycle sequencing kit and CEQ2000 Sequencer, Beckman Coulter). The nucleotide sequences were aligned with the Bestfit program (Smith and Waterman, 1981) revealing four subfamilies: the sequences shown correspond to the first homeobox region of a representative clone of each subfamily. The nucleotides conserved among the 17 cDNA clones are indicated (*).

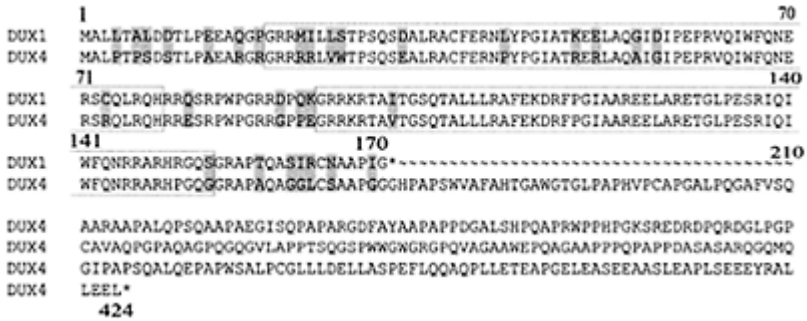

Figure 9.4 Amino acid sequences of DUX1 and DUX4. The two homeodomains of either protein are boxed. Residues differing between DUX1 and DUX4 are shaded grey.

nuclear factors. Two-dimensional gels were performed with extracts of myoblast primary cultures derived from a muscle biopsy of a patient with FSHD whose genotype displayed a short D4Z4 repeat array *(Figure 9.5A)*, and of a non-affected individual *(Figure 9.5B)*. Detection of the low-abundance DUX proteins was performed on a Western blot with the rabbit serum raised against the double homeodomain. About ten protein spots were detected on the gel, among which one was only present in the FSHD sample and corresponded to an isoelectric point (8.6) and an apparent molecular mass (52 kDa) above those predicted from the *DUX4* ORF (pI: 8.5; MW: 45 kDa). As a control, *bona fide*

DUX4 was produced in a TNT reticulocyte lysate (Promega) by transcription/translation of the *DUX4* ORF from a T7 promoter (in the *pCINeoDUX4* vector; Gabriëls *et al.* (1999)) and labelled by

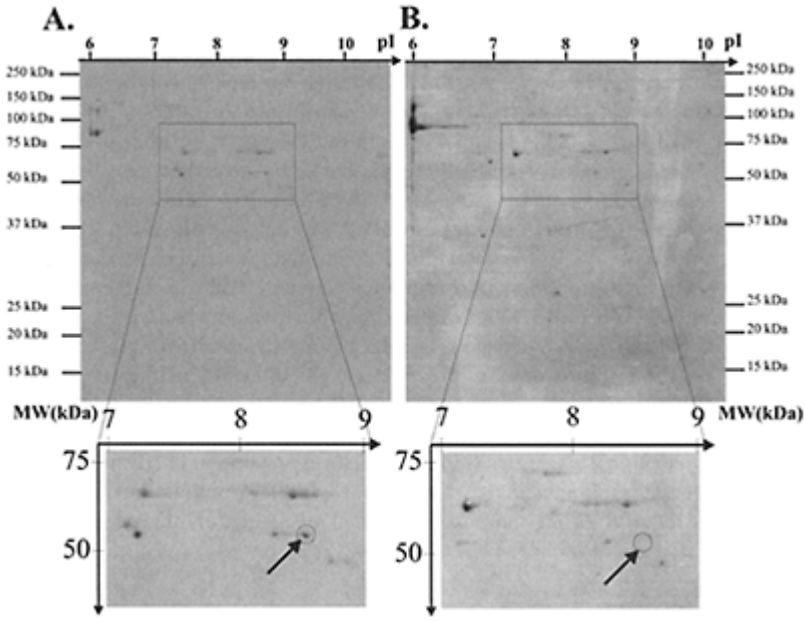

Figure 9.5 Detection of a DUX4 protein in human myoblast. Extracts (100 mg proteins) of primary myoblast cultures derived from (A) a patient with FSHD and (B) a non-affected individual were analysed by IEF (pH range 6–11) followed by electrophoresis on 12% PAGE-SDS. The proteins were transferred from the gel to a polyvinylidene fluoride membrane that was then incubated with a rabbit antiserum raised against the DUX homeodomains. The secondary antibodies (goat anti-rabbit immunoglobulins), coupled to horseradish peroxidase, were detected on a film by production of a chemiluminescent product (ECL Plus

Kit, Amersham). Inserts: focus on the regions where the predicted DUX4 protein would appear. Arrows: protein spot with the expected charge and size of DUX4. Mw: molecular weight markers (kDa). pI: isoelectric point.

incorporation of ^{35}S cysteine. This sample was analysed on a two-dimensional gel run in the same conditions as in *Figure 9.5*. The gel autoradiography revealed a protein spot with a pI of 8.6 and an apparent MW of 52 kDa (data not shown). These results strongly support the hypothesis that the DUX4 protein is expressed in myoblasts of patients with FSHD but not in control myoblasts.

9.7 A function for the DUX4 protein

A nuclear localization signal has been found in the amino terminal part of the paired-type homeodomain in several proteins (e.g. KRKKRR in Vsx1; Kurtzman and Schechter, 2001). A similar stretch of basic amino acids is present in the amino terminal part of the second homeodomain which is the most conserved of all known DUX proteins. Indeed, a fusion of either the full-size DUX1 protein or the DUX4 protein limited to its homeodomains with the green fluorescent protein drove it to the nucleoplasm of transfected C2C12 or TE671 cells (data not shown).

Homeoproteins constitute a large family of transcription factors. DUX1 could indeed specifically bind a DNA sequence and activate transcription of a linked reporter gene in transient co-expression experiments (see Section 9.4; Ding *et al.*, 1998). In order to evaluate a putative DUX4 transcriptional activity we turned to the yeast one hybrid system (reviewed by Vidal and Legrain, 1999). The mutant yeast cells used in this method cannot grow in the absence of histidine or adenine. They can be rescued by expression of the *HIS3* or *ADE2* gene that are activated by the GAL4 transcription factor targeted to a specific *cis*-element in their promoter. The wild-type GAL4 protein comprises a DNA-binding domain and a transcription activation domain. In this assay, the latter domain is replaced by the protein under evaluation: if it presents transcriptional activity it will activate the *HIS3* or *ADE2* promoter. When the DUX4 carboxyl-terminal region was assayed, a strong transcriptional activity was detected, as judged from the number of yeast colonies growing on the selection media *(Figure 9.6A)*. By contrast, neither the DUX4 homeodomains alone (not shown), nor the DUX1 protein *(Figure 9.6B)* exhibited activity in this system. This experiment suggested that the DUX4 protein was a potent transcriptional activator: its inappropriate expression in FSHD might affect several target genes. Interestingly, comparisons of mRNA populations from control and FSHD muscles has demonstrated global misregulation of gene expression (Tupler *et al.*, 1999; Winokur *et al.* 2003).

9.8 A putative DUX4c gene centromeric to D4Z4

The D4Z4 repeat array has a homologue (D10Z10) on chromosome 10q26 which is not linked to FSHD (Deidda *et al.,* 1995). Subtelomeric exchanges between chromosomes 4 and 10 are sometimes observed in patients and can replace 3.3 kb elements of the D4Z4 locus by D10Z10 ones (Lemmers *et al.,* 1998). By computer analysis, we found that the D10Z10 sequence (GenBank Accession #AY028079) contains a *DUX* gene suggesting that subtelomeric rearrangements might simply exchange a *DUX4* for a *DUX10* gene with similar properties. This was confirmed by

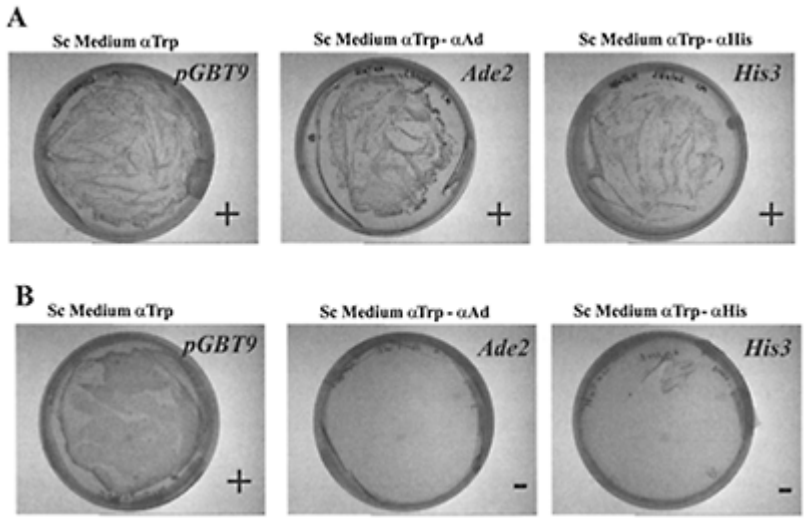

Figure 9.6 Evaluation of DUX4 transcriptional activity by the yeast one hybrid system. *HIS3*$^{-}$, *ADE2*$^{-}$, *TRP* yeast cells were transformed with *pGBT9* vectors expressing the GAL4 DNA-binding domain fused to either the DUX4 carboxyl- terminal domain (A), or the DUX1 full-size protein (B). Colonies containing the *pGBT9* vector (with the *TRP* gene) were selected on medium lacking tryptophane (left panels). Those containing a GAL4 fusion protein able to activate the *ADE2* or *HIS3* gene were detected on

medium lacking adenine (middle panels) or histidine (right panels), respectively.

the study of an FSHD locus resulting from such a telomeric exchange (collaboration with G.Padberg, University of Nijmegen, The Netherlands; R.Frants and S.van der Maarel, University of Leiden, The Netherlands). In precisely comparing this *DUXt10;4* (GenBank Accession #AY044051; Leclercq *et al*, unpublished) and *DUX4* genes, we found sequencing errors in the very GC-rich part located 3′ from the homeoboxes of our previously published *DUX4* sequence (Gabriëls *et al.*, 1999). Their corrections (GenBank Accession #AF1 17653) yielded a 424-codon ORF identical to the *DUXt10; 4* one.

The observation that an identical *DUX* gene is maintained within the FSHD locus upon D4Z4/D10Z10 exchange underscores our hypothesis. However, since the pathology is not observed upon deletion on chromosome 10, one has to consider that chromosome 4 carries unique features that somehow trigger *DUX4* expression. The region of homology between chromosomes 4 and 10 extends from the telomere to the centromere over a distance of about 70 kb (van Geel *et al.*, 2002). By alignment of the 4q35 and 10q26 sequences (GenBank Accession Numbers #AF146191 and AY028079) with the Bestfit program (Smith and Waterman, 1981) the precise centromeric limit of the 4/10 similarity was mapped to about 50 kb from the D4Z4 locus, just centromeric of the recently described *FRG2* gene, and more precisely into a known truncated and inverted D4Z4 unit (locus D4S2463; Wright *et al.*, 1993). It contains a similar putative *DUX* gene that we named *DUX4c (Figure 9.7A)*. We inverted the *DUX4c* sequence for easier comparison with *DUX4 (Figure 9.7B)*. The ORFs and the homeoboxes are perfectly aligned between the two genes which present each a non-canonical TATAA box (TACAA in *DUX4* versus CATAA in *DUX4c)* at a similar position. We showed previously that the TACAA box was functional in the *DUX4* promoter (Gabriëls *et al.*, 1999). Studies in other systems indicated that a TATAA to CATAA mutation did not abrogate promoter activity (Wobbe and Struhl, 1990), suggesting that the *DUX4c* promoter might be functional too. The limit of 4/10 homology maps just upstream of the TACAA/CATAA box, and the coding part of the *DUX4c* gene is missing on chromosome 10. The first 342 amino acids that encompass the double homeodomain and most of the carboxyl-terminal domain are identical in the encoded DUX4 and DUX4c proteins except for two changes (A272P and V279I). DUX4c is 50 residues shorter and the last 32 amino acids are different from DUX4; its theoretical isoelectric point is very high (11.5) in contrast to DUX4 (8.5). Interestingly, one of the cDNA sequences amplified from control human muscle RNA *(Figure 9.4A)* in our attempt at defining *DUX4*-specific primers (see Section 9.6) only presented two mismatches with *DUX4c*, suggesting that this gene might be transcribed *in vivo*.

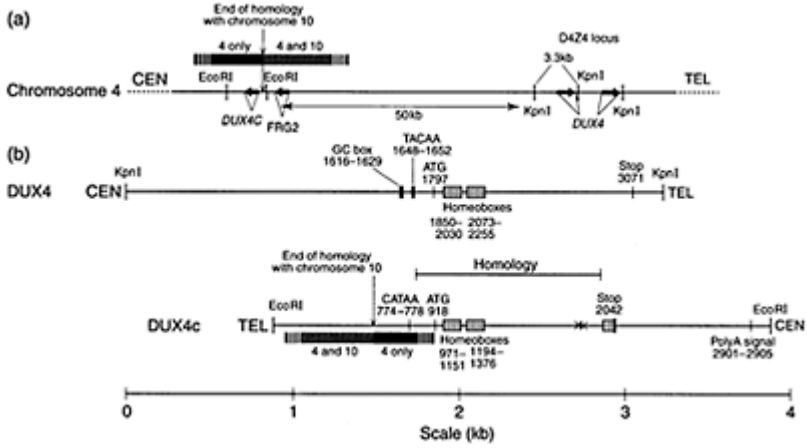

Figure 9.7 (a) Schematic representation (not to scale) of the subtelomeric region of chromosome 4q35 with the positions and orientations of the *DUX4c*, *FRG2* and *DUX4* genes from a patient with FSHD where two copies of the D4Z4 unit are left. (b) Alignment of the *DUX4* and *DUX4c* genes: *DUX4* is shown within a 3.3 kb *Kpn*I fragment corresponding to the D4Z4 unit oriented from the centromere to the telomere. *DUX4c* is shown within a 3.0 kb *Eco*RI fragment 50 kb proximal to D4Z4 and in the opposite orientation. The alignment is based on the homeoboxes: the ORFs (ATG and STOP codons), the sequences of the divergent TATAA boxes, and a putative poly(A) addition signal are indicated. The crosses show mismatches in the *DUX4/DUX4c* sequences affecting the amino acid sequence. The boundary of the chromosome 4 and 10 sequence homology is indicated by a vertical arrow in (a) between the *DUX4c* and

FRG2 genes, and in (b) upstream from the CATAA box.

9.9 Discussion and conclusions

We have discovered a family of genes located within 3.3 kb repeated elements, and encoding double homeodomain proteins. Although these genes present features of pseudogenes, the expression of at least one protein (DUX1) encoded by one such gene *(DUX5)* not linked to FSHD was demonstrated. DUX1 could specifically bind DNA and activate transcription. About 30 other proteins were detected in human myoblast extracts on a two-dimensional gel by Western blotting with a serum raised against the double homeodomain. It was unexpected to discover so many genes outside of the determined human genome sequence, as well as numerous cDNAs lacking in EST databases. The function of these genes/proteins is unknown. However, the similarity with replication-dependent histone genes is intriguing: some are lacking introns and polyA addition signals (Dominski and Marzluff, 1999), they are organized in repeated clusters, the encoded proteins locate to the nucleus, have very high isoelectric points and bind DNA.

We have identified a *DUX4* gene in each D4Z4 unit of the 4q35 locus. In collaboration with Prof. Jane Hewitt (University of Nottingham, UK) we have proposed the following hypothesis as to its role in FSHD. In non-affected individuals the large D4Z4 repeat array would be buried within inhibitory heterochromatin preventing expression of the *DUX4* genes. In patients, this chromatin structure would be destabilized by shortening the D4Z4 array, and could allow expression in some muscle cells of the *DUX4* genes present within the few remaining repeats (Gabriëls *et al.*, 1999).

DNA in the D4Z4 repeats was recently shown to be hypermethylated, as expected for heterochromatic regions (Tsien *et al.*, 2001). The view that the FSHD-linked deletion would lead to a more open chromatin structure and activation of neighbouring genes was recently supported by the experimental data of Gabellini *et al.* (2002). These authors studied the expression of three genes (*FRG2*, van Geel *et al.*, 2002; *FRG1*, van Deutekom *et al.*, 1996; *ANT1*, Wijmenga *et al.*, 1993) located at increasing distances from the D4Z4 locus, and found that the closer a gene was to the D4Z4 repeat array, the more it was repressed in control muscle and 'switched on' in diseased muscle *(Figure 9.8)*. The *DUX4* gene is closer to the D4Z4 array (in fact within it) than any other gene studied so far, and might thus even be more repressed in control muscle, and more activated in diseased muscle than *FRG2*. We have indeed gathered evidence that the DUX4 protein was expressed in myoblasts of patients with FSHD but not in controls *(Figure 9.5)*.

In addition, we have identified a similar *DUX4c* gene located in a truncated and inverted member of the 3.3 kb repeat family 50 kb centromeric to the D4Z4 locus. The homologous subtelomeric regions of chromosomes 4q35 and 10q26 diverge precisely at the *DUX4c* promoter so that its structural part is present on chromosome 4 and lacking on 10. One could speculate that *DUX4c* plays a role in FSHD since shortening of the repeat array is pathological when it takes place on chromosome 4, but not on 10. Based on the expression gradients presented in *Figure 9.8*, *DUX4c* could be expressed at similar levels as *FRG2*, its closest neighbour, i.e. weakly in control muscle, and strongly in FSHD

muscle. The simultaneous accumulation of the DUX4c and DUX4 proteins could lead to the pathology, perhaps

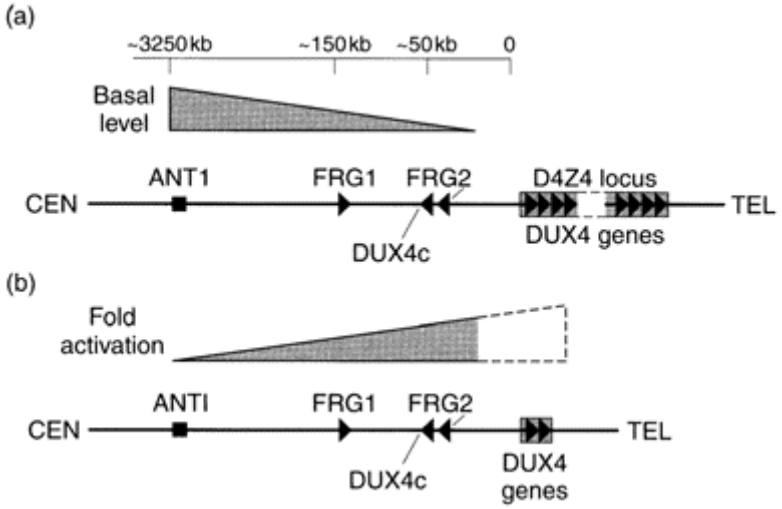

Figure 9.8 Relative location of the *ANT1, FRG1, FRG2, DUX4* and *DUX4c* genes in 4q35. The relative expression levels according to distance from D4Z4 in muscle biopsies of non-affected individuals (a) or patients with FSHD (b) were drawn after data of Gabellini *et al.* (2002). The observed activation gradient suggests that the *DUX4* and *DUX4c* genes are expressed in FSHD muscle cells (see text).

because DUX4 has strong transcriptional activity *(Figure 9.6)* and heterodimerization of these similar proteins would affect expression of some target genes. Another mechanism might involve transcriptional cross-activation of the homologous *DUX4* and *DUX4c* genes. A shortened D4Z4 array could allow intrachromosomal DNA looping and align the two genes in a way similar to the *Figure 9.7B* scheme. This would bring transcriptional activators bound to the *DUX4c cis*-elements into the vicinity of the *DUX4* gene and favour its expression. A similar mechanism was recently shown to allow gene expression in subtelomeric regions of yeast chromosomes (de Bruin *et al.*, 2001).

Several features of FSHD support various aspects of the DUX4 activation hypothesis. First, individuals who presented a complete deletion of all D4Z4 units at 4q35 have been identified, and they did not present with FSHD (Tupler *et al.*, 1996). Second, the dominant inheritance pattern could result from a gain-of-function such as the expression

of a new protein. This new protein might also trigger an immune response thus explaining the notable inflammatory component of muscle pathology in FSHD (Arahata *et al.*, 1995). The disease severity has been negatively correlated with the D4Z4 copy number left (Tawil *et al.*, 1996): with fewer repeat copies, heterochromatin would be easier to 'open' and should therefore allow higher gene expression. The latter point has recently been observed for *FRG2* expression levels (Gabellini *et al.*, 2002). The phenotypic heterogeneity of FSHD might result because heterochromatin 'opening' in position effect variegation is a cell autonomous stochastic event; this is in agreement with previous observations pointing to an epigenetic mechanism such as the typical asymmetrical progression of the disease (Padberg, 1998) and the discordant phenotypes of homozygous twins presenting with the FSHD-associated deletion (Griggs *et al.*, 1995) although a differential inflammatory immune reaction might have contributed to one case (Tupler *et al.*, 1998).

In conclusion, we propose the *DUX4* and *DUX4c* genes as candidates for FSHD and hope that their study will shed light on the molecular mechanism of this pathology.

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10.
Facioscapulohumeral muscular dystrophy (FSHD): a disorder of muscle gene derepression

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10.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common hereditary myopathies with an incidence of 1 in 20 000 in the general population (Padberg, 1982). FSHD onset involves specific facial muscles, i.e. *orbicularis oculi* and *orbicularis ori*, while other muscles are spared, i.e. zygomatic, temporalis and masseter. Subsequently, muscular weakness spreads to limb girdle muscle, such as scapula fixators, and the upper part of deltoid, and to biceps and triceps. Abdominal muscles also become affected leading to a characteristic lordotic posture. Eventually, the disease extends to the muscles of the pelvic girdle. It is important to note that involvement of specific muscle groups leads to a clinical phenotype that is characteristic of FSHD. In addition, weakness displays asymmetric distribution without any correlation with handedness (Brower *et al.*, 1992). Disease progression is unpredictable: dramatic worsening can follow periods of arrest, and considerable clinical variability has been documented. Some subjects experience very mild weakness, being almost unaware of being affected whilst others are wheelchair-dependent even within the same family. Deafness, vascular retinopathy and mental retardation can be ancillary features of the disorder (Padberg *et al.*, 1995; Verhagen *et al.*, 1995; Funakoshi *et al.*, 1998).

Electromyography and histological analysis of muscle tissues reveals non-specific signs of muscular involvement. Thus instrumental examination is not diagnostic; diagnosis of FSHD is mainly clinical.

FSHD is a genetic disorder, transmitted in an autosomal dominant fashion. FSHD1, which defines the major form of FSHD, was mapped to chromosome 4q35 qter by multipoint linkage analysis (Wijmenga *et al.*, 1991; Mathews *et al.*, 1992; Sarfarazi *et al.*, 1992; Upadhyaya *et al.*, 1992). However, evidence of genetic heterogeneity has been

observed in at least 5% of FSHD families (Wijmenga *et al.*, 1991; Gilbert *et al.*, 1992, 1993; Iqbal *et al.*, 1992; Bakker *et al.*, 1995). It is thus expected 0 that at least one additional genetic locus associated with FSHD is present in the genome.

In 1992, Wijmenga *et al.* described the probe p13E-11 that detects in normal individuals polymorphic *Eco*RI alleles usually larger than 35 kilobases (kb). Surprisingly, the same probe revealed *de novo* DNA rearrangements characterized by shorter *Eco*RI fragments ranging between 11 and 35 kb in sporadic FSHD cases, presumably due to a new mutation. The size of the *Eco*RI digested-genomic DNA fragments detected by p13E-1 1 may vary from between 11 kb and 450 kb. Normal subjects carry p13E-11-*Eco*RI alleles larger than 35 kb, whereas alleles shorter than 35 kb are present in the majority of either *de novo* or familial FSHD patients. The *de novo* rearranged short *Eco*RI allele transmitted from an affected parent to their progeny co-segregates with the disorder (Griggs *et al.*, 1993). This observation argued strongly for a causal association between short *Eco*RI alleles and FSHD.

Detailed analysis of the genomic region detected by p13E-l 1 revealed that variation in size observed in normal and FSHD subjects is due to an array of tandemly repeated 3.3 kb segments individually defined by *Kpn*I flanking sites, named hereafter D4Z4 (van Deutekom *et al.*, 1993). The number of D4Z4 3.3 kb units may vary from 11 to 150 in the general population whilst fewer than 11 repeats are present in either sporadic or familial FSHD patients. On chromosome 10q26, a polymorphic region recognized by p13E-11 is 98% homologous to D4Z4. The size of 10q26 alleles varies between 11 and 300 kb, and 10% of these alleles are shorter than 35 kb (Bakker *et al.*, 1995, 1996). The presence of a *Bln*I restriction site within the 3.3 kb *Kpn*I element associated with chromosome 10q allows discrimination between 4q and 10q alleles (Deidda *et al.*, 1996). As a result, Southern blot hybridization of *Eco*RI and *Eco*RI/*Bln*I digested genomic DNA is the currently used tool for the molecular diagnosis of FSHD (Lunt, 1998).

Recombination events between the 4q and 10q subtelomere have been observed. The presence of chromosome 10-type repeats on chromosome 4 and the reverse configuration of chromosome 4-type repeats on chromosome 10 is equally frequent (van Deutekom *et al.*, 1996; van Overveld *et al.*, 2000). Hybrid repeats, consisting of both 4-type and 10-type repeat units, have also been identified. However only short repeat arrays on chromosome 4 are associated with FSHD (Cacurri *et al.*, 1998; Lemmers *et al.*, 1998). These observations are consistent with the view that FSHD is not caused by alteration of the specific sequence composition of the D4Z4 repeats but rather by their structural organization at the 4q35 region.

10.2 D4Z4 contains heterochromatic elements

The D4Z4 unit has a high GC-rich content (Hewitt *et al.*, 1994; Lee *et al.*, 1995) and contains several known sequence motifs: LSau, hhspm3, and a double homeobox domain (Hewitt *et al.*, 1994; Winokur *et al.*, 1994). Lsau is a middle repetitive element associated with heterochromatic regions of the genome (Meneveri *et al.*, 1993) whilst hhspm3 is a low-copy GC-rich repeat (Zhang *et al.*, 1987). Tandemly repeated Sau3A monomers of 68 bp (Agresti *et al.*, 1987; Waye and Willard, 1989) are at the distal end of the D4Z4 arrayed repeats (van Deutekom *et al.*, 1993). Both LSau and β-satellite repeats

preferentially localize to the heterochromatic region of the acrocentric chromosomes, on the pericentric heterochromatin of chromosomes 1, 3 and 9 and on the proximal euchromatic region of the long arm of the Y chromosome (Lyle *et al.*, 1995).

D4Z4 sequence contains a putative promoter with a TACAA box located 149 bp upstream of an 1173 bp ORF (open reading frame) containing two homeodomains (DUX4). Interestingly homeobox domains are known to bind specific DNA sequences and to function as transcriptional regulators during development (Gehring *et al.*, 1990). However, the predicted gene has no introns and lacks a polyadenylation site. No transcript derived from D4Z4 has ever been identified in muscle or other tissues suggesting that is most likely a pseudogene (Gabriels *et al.*, 1999).

Collectively, these data indicated that D4Z4 is a heterochromatic element belonging to a family of 3.3 kb homeobox-containing repeats, subsets of which are clustered at 1q12 and on the short arms of the acrocentric chromosomes.

10.3 The FSHD region on chromosome 4q35 contains genes and pseudogenes

The distal end of chromosome 4q contains the locus involved in FSHD. Despite extensive efforts, identification of candidate gene(s) for FSHD through 'positional cloning' techniques, such as exon-trapping and cDNA selection, has proven very difficult.

The first gene identified within this region was *FRG1* (FSHD Region Gene 1), located approximately 100 kb centromeric to the repeated units (van Deutekom *et al.*, 1996). The 1042 bp transcript consists of nine exons that encode a protein of 258 amino acids with no significant homologies to known genes. The FRG1 protein contains a high proportion of charged amino acids and fascin motif (catalogued as CDD1776 in the protein database at www.ncbi.nlm.nih.gov/Structure/cdd/). Multiple FRG1-related sequences are present in the genome, many of which are considered to be pseudogenes.

A second gene has been identified which maps 80 kb centromeric to D4Z4 on chromosome 4q35. This gene is part of the β-tubulin supergene family and has been designated as TUBB4Q and contains four exons encoding a putative protein of 434 amino acids. The TUBB4Q sequence shares 87% homology to β2-tubulin and TUBB4Q-related sequences are present on multiple chromosomes. However, no transcript derived from TUBB4Q has ever been identified in muscles or other tissues and it has been proposed to be a pseudogene (van Geel *et al.*, 2000).

Recently, a new gene, *FRG2*, mapping 37 kb proximal to D4Z4, has been identified. *FRG2* expression was observed in MyoD-transformed fibroblasts obtained from FSHD patients, while no detectable transcript was present in normal controls (R.R.Frants and S.van der Maarel, personal communication). Multiple *FRG2*-related sequences are present in the genome. As with *FRG1*, its biological function has not yet been elucidated.

A 9.3 Mb contig from the Human Genome Mapping project spans represents the 4q35 chromosomal region. Fourteen known and 53 predicted genes have been identified within that interval. The region 4 Mb centromeric to D4Z4 contains very few genes. Further *FRG1* and *FRG2* are the only genes which have been found to be expressed within this interval. This observation suggests two possible explanations: either the 4q35 region is gene-poor, or genes within this region are normally not expressed, thereby accounting for

the absence of ESTs in the databases. Both possibilities are consistent with the hypothesized heterochromatic nature of this chromosomal region.

10.4 Haploinsufficiency of distal 4q does not cause facioscapulohumeral muscular dystrophy

We described some normal individuals carrying an abnormal chromosome 4 resulting from an unbalanced translocation between the 4q35 subtelomeric region and the short arm of an acrocentric chromosome (Tupler *et al.*, 1996). Based on the restriction map of the distal 4q (van Deutekon *et al.*, 1993), it was possible to locate the breakpoint on the rearranged chromosome 4 and estimate the extent of the deletion. The rearranged chromosome 4 lacks 200 kb containing the entire D4Z4 repeat and the proximal region including *FRG2, TUBB4q* and *FRG1*. This observation indicates that haploinsufficiency of the entire 4q subtelomeric region has no phenotypic consequence, whereas deletion of only the D4Z4 repeats is associated with FSHD.

10.5 Expression of 4q35 genes is up-regulated in FSHD dystrophic muscle

Several lines of evidence, such as the lack of identifiable candidate genes and the causative association of the deletion of repetitive elements with the disease, indicate that FSHD is probably not the result of a classical mutation within a protein-coding gene. Instead, the genomic organization of the 4q35 subtelomeric region strongly argues for its role in the control of gene expression.

The hypothesis that genes within this region are abnormally expressed provided the basis of a new model which proposes that the inappropriate silencing of 4q35 genes is due to a positional effect emanating from the repeated elements. Thus, one immediate prediction of the model is that deletions of D4Z4 would place 4q35 genes under the control of telomeric heterochromatin, thereby causing their transcriptional silencing (Wijmenga *et al.*, 1992; Hewitt *et al.*, 1994; Winokur *et al.*, 1994; Fisher and Upadhyaya, 1997). However, several observations did not fit with this hypothesis. First, no genes within the 100 kb region proximal to the D4Z4 repeat have been found to be expressed in normal tissues (van Deutekom *et al.*, 1995). Second, haploinsufficiency of a 200 kb region including the D4Z4 repeat has no phenotypic consequences indicating that the reduced expression of 4q35 genes does not cause FSHD (Tupler *et al.*, 1996). Third, increasing evidence indicates that it is the expansion of repeated elements or long tandemly arrayed sequences—not the reduction of these elements—that is associated with suppression of transcription (Dorer *et al.*, 1994; Garrick *et al.*, 1998; Saveliev *et al.*, 2003).

An alternative model for the molecular basis of FSHD proposed instead that the D4Z4 deletion might induce abnormal transcriptional activity of the 4q35 genes. This hypothesis was supported by several observations. First, D4Z4 displays features common to heterochromatic elements, in that it has a high CG content (Hewitt *et al.*, 1994) and is hypermethylated in normal tissues (Tsien *et al.*, 2001). Thus, one prediction would be

that the reduction of D4Z4 element number derepresses gene transcription. Second, all approaches aimed at identifying genes within the region, such as exon trapping or cDNA selection or bioinformatic analysis have failed, leading to the only possible conclusion that the 4q35 region is either gene-poor, or that genes within this region are not expressed in most normal fetal and adult tissues and, therefore, are under-represented in commercial cDNA libraries.

Confident that the latter hypothesis was correct, we carried out a differential mRNA screen on normal and FSHD muscle. The results although complex, clearly indicated that the transcriptional profile of FSHD dystrophic muscle is profoundly different from that of normal muscle. Intriguingly, several transcriptional regulators are either up- or down-regulated in the affected muscle tissue, suggesting that a general transcription regulatory defect might be the underlying basis of the FSHD dystrophic process (Tupler *et al.*, 1999). This hypothesis has been further strengthened by recent work showing that the expression level of known 4q35 genes such as *FRG2*, *FRG1* and *ANT1* is specifically elevated in FSHD dystrophic muscle (Gabellini *et al.*, 2002).

Indeed, the expression of *FRG2*, the gene most proximal to D4Z4, was undetectable in normal muscle but was present at a significant level in all FSHD muscle samples analysed. Expression of two other 4q35 genes, *FRG1* and *ANT1*, was detected in all muscle samples but the genes were overexpressed only in FSHD muscle. Extending the analysis to human muscle samples from patients affected by other muscular dystrophies confirmed the specificity of overexpression observed in FSHD muscle. Significantly, in lymphocytes from FSHD patients, expression of *FRG2*, *FRG1* and *ANT1* was equivalent to that observed in normal tissue, indicating that overexpression of 4q35 genes in FSHD may be muscle-specific. Interestingly, the expression level of *FRG2* ascertained in FSHD patients carrying varying number of D4Z4 repeats was found to be inversely related to the number of D4Z4 repeats. These data are consistent with the observation that the number of D4Z4 repeats is a critical determinant of disease severity (Ricci *et al.*, 1999).

10.6 Detection of a nuclear activity that binds to D4Z4

Analysis of the interaction between D4Z4 and nuclear proteins using an electrophoretic mobility shift assay (EMSA) showed that one DNA fragment, termed D4Z4-243, supported formation of a specific complex. This complex was abolished by addition of increasing amounts of D4Z4-243 DNA but not by non-specific DNA. A comparable D4Z4-243 binding activity was also detected in nuclear extracts prepared from several human and mouse myogenic cell lines (Gabellini *et al.*, 2002).

Deoxyribonuclease I (DNase I) footprinting was used to map the binding site within D4Z4-243. It revealed that a 27 basepair (bp) sequence within D4Z4-243 was protected from DNase I digestion and has been termed D4Z4 Binding Element (DBE). A colony assay showed that multimers of the DBE can suppress expression from an upstream promoter, leading to the conclusion that the DBE within D4Z4 is a transcriptional repression element. Biochemical studies demonstrated that the DBE binds a multiprotein complex formed by YY1, HMGB2 and nucleolin (Gabellini *et al.*, 2002).

YY1 is a complex protein that is involved in repressing and activating a number of promoters. It interacts with numerous key regulatory proteins suggesting that these

interactions are important for determining which particular function of YY1 is displayed at specific promoters (Thomas and Seto, 1999). Furthermore, histone deacetylases and histone acetyltransferases modulate YYI's function. These two groups of enzymes modify histones and this modification is proposed to alter chromatin structure. Interestingly, the DBE contains a putative YY1 recognition sequence (CCATN; Yant *et al.*, 1995), and EMSA experiments have suggested that YY1 has an intrinsic DBE-binding activity and that YY1 is the most likely D4Z4 recognition complex (DRC) component to directly interact with the DBE. *In vivo* and *in vitro* experiments demonstrated that the YY1, HMGB2 and nucleolin multiprotein complex was in fact the nuclear DBE-binding activity (Gabellini *et al.*, 2002).

HMGB2 is a member of one of the three families of high mobility group (HMG) proteins (for reviews, see Bustin, 1999; Bianchi and Beltrame, 2000; Agresti and Bianchi, 2003). The family comprises the ubiquitous Hmgb1, Hmgb2 and Hmgb3 proteins and is known as HMGB. Hmgb1 is the most studied member of this family. It interacts with the minor groove of DNA and bends the DNA segment to which it is bound. Although it does not exhibit sequence specificity, HMGB1 binds with high affinity to unusual DNA structures like four-way junctions and DNA bulges and can be recruited via protein-protein interactions by a variety of sequence-specific DNA-binding proteins thereby facilitating the assembly of multiprotein complexes on DNA. It is therefore considered to have an 'architectural' function.

Hmgb2 is extremely similar to Hmgb1 (more than 80% amino acid identity). It has been shown that Hmgb1 and Hmgb2 are completely interchangeable *in vitro:* they both bind to Hox proteins (Zappavigna *et al.*, 1996), steroid hormone receptors (Booyaaratanakornkit *et al.*, 1998) and Rag1 recombinase (Aidinis *et al.*, 1999) and both enhance the transcription and recombination activities of their partner proteins. However, knockout experiments indicate that the two proteins have non-redundant biological functions *in vivo* (Calogero *et al.*, 1999; Ronfani *et al.*, 2001). A yeast two-hybrid screen identified SP100 as an interactor of HMGB2; SP100 in turn interacts with HP1, the cardinal heterochromatin-binding protein, raising the possibility that HMGB2 might be involved in the organization and/or maintenance of heterochromatic regions (Lehming *et al.*, 1998).

Nucleolin is an abundant protein in the nucleolus. The association of several structural domains in nucleolin allows the interaction of nucleolin with different proteins and RNA sequences. Nucleolin has been implicated in chromatin structure, rRNA transcription, rRNA maturation, ribosome assembly and nucleocytoplasmic transport (for a review, see Ginisty *et al.*, 1999).

Since the human genome contains sequences homologous to D4Z4 on several chromosomes in addition to chromosome 4 (Wijmenga *et al.*, 1992; Winokur *et al.*, 1994; Lyle *et al.*, 1995), it was necessary to confirm that the interaction between the DRC and DBE occurs at 4q35 rather than at one or more of the other chromosomal loci. Since the rodent genomes lack D4Z4 repetitive sequences (Clark *et al.*, 1996), chromatin immunoprecipitation (ChIP) experiments were performed in human/rodent monochromosomal cell hybrids containing a single human chromosome 4. These experiments confirmed that YY1, HMGB2 and nucleolin, were associated with D4Z4 sequences at 4q35.

Antisense experiments to decrease the intracellular levels of DRC components have demonstrated that depletion of YY1, HMGB2 or nucleolin results in overexpression of the 4q35 gene *FRG2*, which has been shown to be silent in normal cells and tissues. Thus, reducing the levels of DRC components recapitulates the molecular event observed at 4q35 in FSHD muscle.

Consistent with this observation, we have discovered that HMGB2 is part of the transcriptional repressor complex-binding D4Z4; down-regulation of HMGB2, as well as nucleolin or YY1, by antisense oligonucleotides can relieve silencing at 4q35.

All these observations suggest a model in which deletion of D4Z4, as it occurs in FSHD patients, leads to the inappropriate transcriptional derepression of 4q35 genes, resulting in disease.

10.7 A new model for FSHD

For a long time, FSHD has been a hereditary disorder in search of a genetic defect. The recent results described above suggest a new model for the molecular basis of FSHD and a novel mechanism for human genetic disease *(Figure 10.1A)*. In normal individuals, the presence of a threshold number of D4Z4 repeats leads to repression of 4q35 genes by virtue of a DNA-bound multiprotein complex that actively suppresses gene expression. In FSHD patients, deletion of an integral number of D4Z4 repeats reduces the number of bound repressor complexes and consequently decreases (or abolishes) transcriptional repression of 4q35 genes. As a result, these genes are inappropriately overexpressed, ultimately leading to the onset of disease.

The derepression model is consistent with the clinical and genetic features of FSHD as follows:

1. Gene overexpression provides a molecular explanation for the autosomal-dominant transmission of FSHD.
2. The observation that the 4q35 gene overexpression is muscle-specific accounts for the apparent tissue specificity of the disease.
3. The D4Z4 deletion may result in variegated expression of 4q35 genes in FSHD muscle cells, by a mechanism similar to position effect. This stochastic variation in gene expression in muscle cells can explain the asymmetric involvement of muscle and the great variability of clinical expression between and within families. It can also account for the apparent threshold effect whereby there is a requirement for the deletion of a certain number of copies of D4Z4.
4. In addition to overexpression of 4q35 genes, factors such as the allelic variability of 4q35 genes, gender and environment that might interfere with the activity of DRC can potentially explain the significant variability in disease onset and clinical severity observed in FSHD patients, even within the same family.

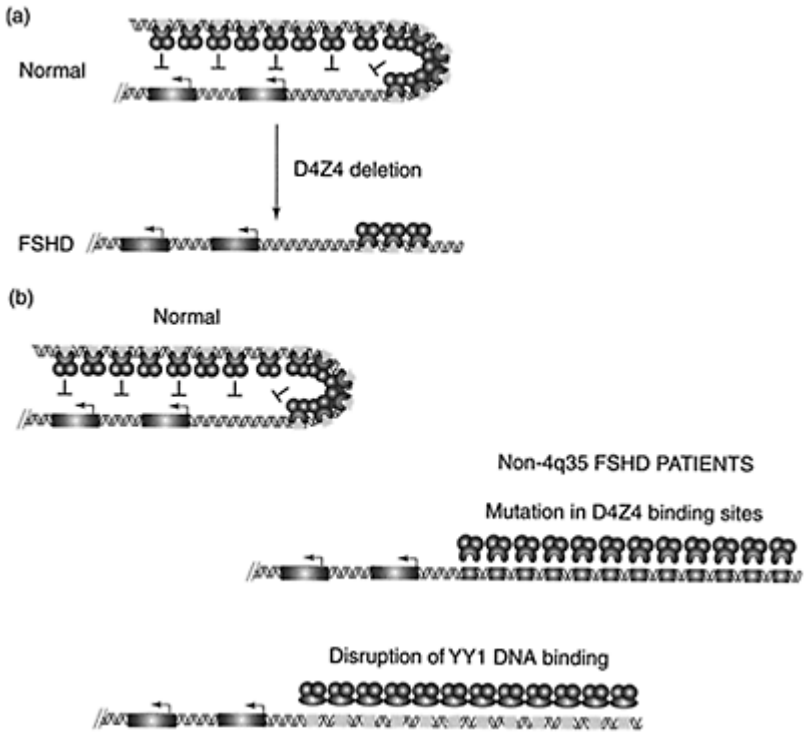

Figure 10.1 (A) In normal individuals, the presence of a threshold number of D4Z4 repeats leads to repression of 4q35 genes by virtue of a DNA-bound multiprotein complex that actively suppresses gene expression. In FSHD patients, deletion of an integral number of D4Z4 repeats reduces the number of bound repressor complexes and consequently decreases (or abolishes) transcriptional repression of 4q35 genes. As a result, these genes are inappropriately overexpressed, ultimately leading to the onset of disease. (B) The proposed model might also explain the disease onset in the 5–10% of FSHD cases that are not linked to the 4q35 locus. One possibility is

that mutations in either the DBE sequence or one of the genes encoding a DRC might lower the activity of the repressor complex, thereby triggering the derepression of *cis*-linked genes and eventually the disease.

The model proposed suggests that deletion of repeated elements in the subtelomeric region of 4q may act in *cis* on neighbouring genes by derepressing their transcription thereby initiating a cascade of events that ultimately leads to FSHD. This idea is consistent with the observation that haploinsufficiency of distal 4q does not cause FSHD (Tupler *et al.*, 1996).

The proposed model might also explain the disease onset in the 5–10% of FSHD cases that are not linked to the 4q35 locus. One possibility is that mutations in either the DBE sequence or one of the genes encoding a DRC might lower the activity of the repressor complex, thereby triggering the derepression of *cis*-linked genes and eventually the disease *(Figure 10.1B)*. Furthermore, this model can explain sporadic and familial FSHD cases with no D4Z4 deletion and can fit both dominant and recessive modes of inheritance. Consistent with the proposed model, sporadic patients might arise as a consequence of the *de novo* occurrence of dominant mutations, but also through the inheritance of recessive alleles from both parents.

In conclusion, FSHD pathogenesis provides a new paradigm for dominantly inherited genetic disease by demonstrating that repetitive sequence elements, which represent a large portion of the human genome, can play an important role in gene regulation.

10.8 Open questions

The model proposed to explain the pathogenesis of FSHD indicates that deletion of repeated elements in the subtelomeric region of 4q causes FSHD through the transcriptional derepression of neighbouring genes. The molecular mechanism proposed has addressed several questions. However, the mechanistic basis by which the DRC represses transcription in a tissue-specific manner remains to be determined.

Several factors or molecular mechanisms may contribute either independently or cooperatively to this phenomenon. For example, the components of the DRC might possess a certain activity level only in muscle cells which may be particularly sensitive to the loss of repressor function. It is also possible that other regulatory proteins could confer tissue-specific activity upon the DRC.

Gene activation, observed in FSHD dystrophic muscle, involves only a subset of genes located at 4q35. It is thus plausible that tissue-specific transcription factors could promote the specific expression of 4q35 genes in muscle tissue; as a consequence, in the absence of a functional repressor, these genes would be expressed. Another possibility is that at 4q35, chromatin might have a more relaxed conformation in muscle cells, allowing for tissue-specific differences in the expression of local genes. These possibilities are not mutually exclusive. Elucidation of these aspects of FSHD pathophysiology will provide

important information that will help us to understand the complex mechanisms controlling tissue-specific gene expression.

It is also possible that D4Z4 deletions might affect 4q35 gene expression through modification of higher-order chromatin structure. Patterns of gene expression that are established and maintained during cellular differentiation might result not only from the targeting of stage-specific transcription factors, but can also be influenced by the long-range organization of chromatin, which establishes 'open' or 'closed' conformations that are permissive or refractory to transcription. In yeast, it has been shown that both the concentration of general chromatin factors and their subnuclear distribution play crucial roles in chromatin-mediated gene regulation. In *S. cerevisiae,* genes inserted at telomeres become repressed through their association with a complex of three silent information regulators (Sir3p and Sir4p), which can bind the N termini of histones H3 and H4 and propagate along nucleosomes (for a review, see Grunstein, 1998). Clearly, it is the clustering of yeast telomeric repeats that promotes the concentration of Sir proteins, and not *vice-versa* (Gotta *et al.,* 1996(Q2)). It has been hypothesized that Sir proteins and transcriptional activators compete for a given promoter once it is within a compartment that can confer repression, such as that formed by telomeric foci (Aparicio and Gottschling, 1994). Although Sir proteins are concentrated at telomeres, increased expression can improve silencing efficiency, indicating that the normal protein levels are limiting for repression. It would be interesting to determine whether deletions of the D4Z4 repeats affect the global structural organization of 4q subtelomeric heterochromatin.

It is interesting to note that repression at 4q35 fulfils the criteria proposed to define the heterochromatin-type repression proposed by Gasser (2001). These criteria include the identification of structural chromatin factor such as HMGB2; complexes which are bound within the nucleus via a sequence-specific DNA-binding factor such as YY1, which is, as expected, implicated in both transcriptional activation and repression, depending on the context. Moreover, multiple YY1-binding sites are present in repetitive DNA.

It is therefore possible that the DRC mediates a 'closed' higher-order chromatin structure, a phenomenon that has been observed with Polycomb group (PcG) proteins, which control the expression of important developmental regulators. YY1 has sequence homology to the *Drosophila* Polycomb Group protein, pleiomeiotic (PHO) (Brown *et al.,* 1998) and the DBE contains a putative Polycomb Response Element. The homology between YY1 and *Drosophila* PHO resides in two YY1 domains, suggesting that YY1 might be a vertebrate counterpart of PHO and thus could function as a PcG protein. Recent experiments, carried out in *Drosophila,* show that YY1 stably represses transcription on a PcG-responsive promoter, but not on a PcG-non-responsive promoter, whilst it functionally compensates for loss of PHO in pho mutant flies and partially corrects mutant phenotypes (Atchison *et al.,* 2003). This observation is particularly intriguing since PcG proteins interact with many genes in *Drosophila* as well in vertebrates. In particular, they establish the silent state of homeotic genes in *Drosophila* shortly after gastrulation. More remarkably, the PcG protein complexes preserve a memory of the early state of activity through many rounds of cell division. It would be interesting to establish whether the DRC shares functional homologies with PcG protein complexes. Consequently, FSHD represents a valuable model for studying the role of

chromatin conformation and nuclear position in the control of tissue-specific gene expression.

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11.
Genotype-phenotype relationships in FSHD

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11.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is characterized by wide variation in clinical expression, both between and within families (Lunt and Harper, 1991). At its most severe there is proximal lower limb as well as upper limb and facial weakness from early childhood giving rise to a requirement for a wheelchair while still in teenage years (Lunt *et al.*, 1995a). By contrast, there are affected subjects in their 70s who may have only minimal facial and shoulder girdle weakness, while a significant proportion of gene carriers, particularly women, may remain asymptomatic (Zatz *et al.*, 1998). Within individual families the extremes of presentation rarely occur together. The one exception to this is where the origin of a new mutation may have been in postzygotic mitosis of the parent of a severely affected child, such that the parent manifests the same DNA mutation in only a proportion of their cells (somatic mosaicism). Thus, it is suggested that there is a likelihood of a link between phenotype and genotype.

This chapter assumes that the reader is familiar with the molecular mechanism underlying FSHD: i.e. the deletion of integral numbers of copies of 3.3 kb D4Z4 repeats at 4q35 (van Deutekom *et al.*, 1993); the close homology with similar repeats at 10q26 and the use and limitations of the *Bln*I restriction site to aid their differentiation (van Deutekom *et al.*, 1996). There is evidence, principally in two large families from the USA, supporting the concept of a second gene locus, giving rise to a condition (FSHD1B) clinically indistinguishable from FSHD1A (Gilbert *et al.*, 1993). No gene, or even chromosomal location, has yet been identified for these two families (Gilbert *et al.*, 1995). They are not therefore discussed further in this chapter, which concentrates exclusively on FSHD1A.

11.2 What are the variations in phenotype and genotype against which any relationship between these can be gauged?

11.2.1 Phenotypic parameters studied in FSHD

1. Distribution of muscle involvement.
2. Clinical asymmetry, handedness.
3. Pattern of progression of muscle involvement.
4. Severity of muscle symptoms.

   – objective measures of muscle strength
   – age at first requirement for wheelchair
   – broader functional categories of disability: ‘mild’, ‘moderate’, ‘severe’.

5. Age at onset:

   – of recognized symptoms
   – of clinical signs.

6. Possible associated features:

   – hearing loss
   – retinal vascular telangiectasis
   – cardiac involvement
   – learning difficulty
   – epilepsy
   – tongue atrophy.

7. Clinical non-penetrance (gene carriers without clinical signs or symptoms).
8. Clinical anticipation (which remains controversial and unproven).
9. Extent of known family history of FSHD.
10. Serum creatine kinase (CK).
11. Muscle biopsy features: e.g. inflammation, neurogenic and/or myopathic pattern.

11.2.2 Genotypic parameters against which the phenotypic parameters can be assessed

1. The presence (versus absence) of a typical molecular diagnostic finding (i.e. proven D4Z4 copy deletions at 4q35 leaving a residual number of *Eco*RI/*Bln*I-resistant copies in accepted range for causing FSHD).
2. Size of residual fragment at 4q35 (i.e. number of copies of repeat remaining).
3. Atypical DNA finding present, which might be:

   – no shortened *Eco*RI fragment detected by p13E-11, due to deletion of the p13E-11 hybridization site (shown by using different probes)
   – shortened *Bln*I-sensitive fragment proven to be on 4q35

– shortened *Bln*I-sensitive fragment, but not known whether on 4q35 or 10q26
– *Bln*I-resistant fragment above normal accepted limit of size range for FSHD: i.e. having 9–13 repeats (35–48 kb residual DNA fragment)
–ther unusual DNA finding (e.g. two *Bln*I-resistant fragments).

4. Somatic mosaic for typical FSHD deletion.
5. Proven *de novo* mutation for typical FSHD deletion (neither parent has the same fragment, paternity proven).
6. Male versus female patient.
7. Sex of transmitting parent or of parent of origin of mutation.
8. Size or character of non-affected 4q35-allele or of repeat arrays on 10q26.
9. Homozygosity for typical mutation or for atypical mutation.
10. 4q35-telomeric polymorphic segment (4qA or 4qB).

Not all of these genotypic variations can be assessed in a laboratory offering molecular diagnostic testing for FSHD as a service. They are listed here as the relevant genotypic parameters which have been studied on a research basis to help to explain phenotypic variation. Only some of the potential cross-correlations between genotypic and phenotypic parameters have been studied, but few of the parameters are independent within each group. Broad correlations between phenotype and genotype are observed, which are proving very helpful in the interpretation of diagnostic molecular tests, and for meaningful clinical genetic counselling. However, there are also emerging trends for exceptions to these correlations (Butz *et al.,* 2003) that will help advance our understanding of the links between phenotype and genotype in FSHD.

11.3 Correlations and exceptions

11.3.1 The broad correlations

1. There is increased clinical effect (increased ‘severity’ or earlier ‘age at onset’) with lower residual D4Z4 copy number (smaller residual fragment size) (Lunt *et al.,* 1995a, 1995b; Zatz *et al,* 1995; Tawil *et al.,* 1996).
2. It remains uncertain as to whether there may also be a greater likelihood of the associated clinical problems (e.g. significant hearing loss, or Coats’-like retinal changes) with fewer residual D4Z4 copies (Padberg *et al.,* 1995; Rogers *et al.,* 2002). Certainly, those patients reported with epilepsy and/or a learning difficulty have been those with early childhood onset and the fewest possible (one) residual D4Z4 copy number (10–11 kb fragment) (Funakoshi *et al.,* 1998).
3. New mutation or mosaic cases tend to manifest greater clinical severity and smaller residual fragment size than familial cases (Lunt *et al.,* 1995a; Zatz *et al.,* 1998; Orrell *et al.,* 1999; van der Maarel *et al.,* 2000).
4. With larger residual fragment sizes, new mutation is very rare, and most apparently ‘isolated’ cases will be associated with significant familial non-penetrance (Lunt *et al.,* 1995b; Busse *et al.,* 2000; Lunt, 2000; Tonini *et al.,* 2004).

5. The clinical effect in males tends to be greater than in females and a higher proportion of female heterozygotes can show non-penetrance (Padberg, 1998; Zatz *et al.,* 1998; van der Maarel *et al.,* 2000).
6. Mosaic cases due to somatic new mutation are more likely to be symptomatic if they are male, and with a lower number of residual repeats (van der Maarel *et al.,* 2000).
7. In a molecular diagnostic setting, personal experience suggests that the clinical variation for patients with *Bln*I-sensitive fragments only, or with no small *Eco*RI fragment, is very wide, such that most of these patients will have alternative diagnoses.
8. FSHD with a typical clinical presentation and molecular findings appears to be associated exclusively with the type A polymorphic allele at the 4q telomere (Lemmers *et al.,* 2002). Note that it is the 4qA allele which seems to be invariant at the equivalent region on 10q26.
9. The occurrence of a 4q-type *Bln*I-resistant repeat array at 10q26 may predipose to new mutation for deletion of typical *Bln*I-resistant repeats at 4q35, and hence to new FSHD mutation (van der Maarel *et al.,* 2000).

11.3.2 Emerging exceptions/cautions

1. A few cases have been observed of an asymptomatic parent of a clinically apparent new mutation and severely affected child appearing to have the same *Bln*I-resistant small residual fragment, but without any reduction of band intensity which might have suggested mosaicism (Lunt *et al.,* 1998).
2. The inverse correlation between clinical effect and fragment size seems to apply less well in some patients or families with fragment size towards and above the normally considered upper limit for diagnosis of FSHD (i.e. ≥8 repeats, or above 32 kb)(Butz *et al.,* 2003).

11.3.3 Null effects

1. The course of progression of FSHD (i.e. whether peroneal muscles are affected before pelvic muscles) does not seem to correlate within a family nor with residual fragment size (Padberg, 1998).
2. Somatic mutation seems equally likely to occur in males or females, although males may be more likely to be symptomatic themselves (van der Maarel *et al.,* 2000).
3. The parent of origin (of a familial case or a new mutation) probably does not influence the clinical effect in affected offspring *per se,* and an apparent greater severity in offspring with maternal rather than paternal origin of the mutation (Zatz *et al.,* 1995; Kohler *et al.,* 1996; Upadhyaya *et al.,* 1995), may be better explained by effects on ascertainment bias consequent to the tendency for milder presentation in females (Zatz *et al.,* 1998; van der Maarel *et al.,* 2000).

11.3.4 Unknowns

1. Does the clinical effect or another aspect of the phenotype of non-typical deletions at 4q35 (e.g. *Bln*I-sensitive repeats, p13E-11 probe deletion cases) differ in any way from standard FSHD (Köhler *et al.,* 1996)?

2. Does a short *Bln*I-sensitive repeat array on 4q35 that would be predicted to cause FSHD, show the same exclusivity with the 4qA telomere polymorphic allele.

3. Does a 4qB telomere polymorphic allele at 4q35 protect against repeat array deletion, or would this lead to a different phenotype? Actually the most recent evidence suggests that a deletion on the background of a B-type polymorphism can occur, but has no clinical effect and does not cause FSHD (van der Maarel *et al.,* 2003).

4. What is the clinical phenotype associated with homozygosity for the typical FSHD deletion?

5. Is there a phenotype associated with homozygosity for a 10q26 *Bln*I-sensitive repeat array deletion?

6. Is there co-localization/pairing or other interaction between type A 4q35 and 10q26 telomeres in mitotic (or meiotic) cell division which may predispose to somatic (or germinal) mutation for FSHD?

7. Is there incontravertable evidence for clinical anticipation in FSHD (Lunt *et al.,* 1995a; Zatz *et al.,* 1995; Tawil *et al.,* 1996) at least in some families?

11.4 Evidence for correlations

11.4.1 Increasing clinical effect with reducing residual fragment size

This has now been observed by several different research groups and in all different populations studied worldwide (Jardine *et al.,* 1994b; Lunt *et al.,* 1995a, 1995b; Zatz *et al.,* 1995; Tawil *et al.,* 1996; Padberg, 1998), whether using age at first onset, age at requirement for a wheelchair, or direct clinical severity as the parameter to measure clinical effect. However, the correlation only really holds for the difference between the most severe presentations of FSHD, usually seen in early-onset new mutation cases, and the more typical moderate and mild cases, often from large extended pedigrees. Within familial cases alone, any correlation of severity with fragment size is masked by the considerable variation of clinical severity within families (Rogers *et al.,* 2002; Butz *et al.,* 2003; Tonini *et al.,* 2004). Current evidence suggests that for the largest fragment sizes (>8 repeats or 32 kb) the correlation may cease to exist (Butz *et al.,* 2003). However, whether this is a real effect, or whether it is merely an apparent effect resulting from

reduced ascertainment of asymptomatic gene carriers at the larger fragment sizes, is not known. Families in which many gene carriers are asymptomatic may tend to be under-ascertained, or, unless comprehensive family molecular testing is pursued, may be thought to comprise only the one or two gene carriers who happen to have very obvious symptoms.

At the upper end of the range of fragment size that can be associated with clinical symptoms (i.e. 9–11 repeats or 35–42 kb), it seems likely that the FSHD population merges with the normal population. Whether someone with a fragment in this size range manifests any clinical symptoms is not readily predictable, but appears likely to be subject to decreasing probability for increasing residual fragment size. New mutations for fragments in this size range have rarely, if ever, been reported. It is expected that most symptomatic subjects with residual repeat copy numbers in this size range, would, if family testing allows, be shown to have many relatives who have inherited the same fragment whilst remaining asymptomatic (i.e. penetrance becomes very low). Therefore, most pedigrees with these larger fragments appear to be small (Lunt *et al.,* 1995b), but DNA testing has not really been pursued through the families. Some of the subjects who are symptomatic have been found to show a predominantly scapulohumeral presentation with no characteristically detectable facial weakness, but with this pattern consistent through a family (Jardine *et al.,* 1994a; Butz *et al.,* 2003). However, in some other families the presentation has been a more classical FSHD picture (Butz *et al.,* 2003). This can lead to difficulties in the interpretation of molecular genetic results when a single DNA sample from a patient with some muscle weakness is received by a test laboratory for diagnostic testing for FSHD.

A large size (9–11 repeats or 35–42 kb) *Bln*I-resistant fragment, may indicate an expected mild presentation of FSHD. However, if the patient is an apparently isolated case of a relatively severe neuromuscular condition, a *Bln*I-resistant DNA fragment of this size is more likely to be coincidental rather than causative, and the suggestion of FSHD as the diagnosis may be erroneous and misleading. Usually then the fragment would be due to the near-homologous 3.3 kb repeats on chromosome region 10q26 being of 4q-type (*Bln*I-resistant) rather than 10q-type (*Bln*I-sensitive), but therefore unrelated to FSHD (van Deutekom *et al.,* 1996).

If the neuromuscular presentation is typical for FSHD but is significantly more severe than might be expected from the fragment size, the possibility that there might be a separate FSHD-causing D4Z4 deletion (i.e. deleting most of the repeats) but which encompasses also the p13E-11 probe hybridization site and therefore is undetected (while the observed fragment is coincidental and originates from 10q) should be considered (Lemmers *et al.,* 1998). This can be tested for by using a probe complementary to the D4Z4 sequence itself, or telomeric to this (Lemmers *et al.,* 1998, 2003).

Sometimes the clinical presentation may be much milder than expected from the fragment size. If so, the possibility of somatic mosaicism for a *de novo* mutation should be considered and tested for as appropriate (van der Maarel *et al.,* 2000).

11.4.2 Infantile-onset cases and wider clinical effects

Ever since the recognition of cases with a very early onset of symptoms of FSHD, it had been uncertain whether this ‘infantile-onset’ form represented the same or a different

condition. Invariably, cases appeared to be isolated and *de novo*, but exceptions have been noted, with occasional recurrence in sibs due to parental somatic and germinal mosaicism (discussed below). The identification of the D4Z4 deletions in these cases has confirmed that they do not have a condition distinct from the more typical familial FSHD: rather, they represent one end of the spectrum of the condition. The correlation between severity and fragment size is particularly pertinent in infantile cases, who will almost always have an associated very small residual fragment of only 1–3 residual repeat copies (7–14 kb residual *Bln*I-resistant DNA fragment) (Jardine *et al.*, 1994b; Brouwer *et al.*, 1995; Lunt *et al.*, 1995a; Funakoshi *et al.*, 1998).

Additional clinical features have been associated in some of the most severe infantile-onset cases, including: sensorineural hearing loss (Small, 1968; Jardine *et al.*, 1994b), retinal vascular telangiectasis presenting similar to Coats' disease (Small, 1968; Gurwin *et al.*, 1985) tongue atrophy (Yamanaka *et al.*, 2001), epilepsy and learning difficulties (Funakoshi *et al.*, 1998). Any suggestion of apparent learning difficulty must be distinguished from the potential effects on early childhood communication and social interaction of a combination of hearing loss and expressionless myopathic face. However, in view of additional anecdotal examples known to the author, the possibility of true learning difficulty occurring as a pleiotropic effect of the deletion in the most severe infantile-onset cases with no more than two residual D4Z4 repeats, still remains.

Models for the mechanism of action of the mutation and of affected genes (discussed below) will need to account for these pleiotropic effects, and for the observation that they seem more likely to occur with the largest deletions (smallest residual repeat copy number). However, reports of retinal vascular problems have also been based on family members from more typical families (Fitzsimons *et al.*, 1987; Padberg *et al.*, 1995), now known to have a more standard residual repeat copy number of around 5–6 repeats (22–28 kb fragment).

11.4.3 Male:female difference

Several clinical studies have looked for evidence for differences in age at onset or severity between males and females (Padberg, 1982; Lunt *et al.*, 1989; Lunt and Harper, 1991; Zatz *et al.*, 1998). Overall it is apparent that males tend on average to have an earlier age at onset, and to be more severely affected than females, at least up to age 50 years (Padberg 1998; Zatz *et al.*, 1998). With a greater number of residual D4Z4 copies (larger residual fragment sizes), a greater proportion of adult females than males of the same age may be non-manifesting 'carriers' (Padberg, 1998; Zatz *et al.*, 1998; Tonini *et al.*, 2004). In cases who are somatic mosaics (see below) it is found that for any given size of mutation (i.e. for any given number of residual repeats or residual fragment size), a smaller percentage of cells with the mutation is required for a male to be symptomatic than for a female (van der Maarel *et al.*, 2000). Thus, mosaic males tend to present as the affected index new mutation case, whereas mosaic females tend more to be found as asymptomatic parents of an apparently first, but usually severe (see below), case in a family (van der Maarel *et al.*, 2000). Explanations for the male/female differences can at present only really be speculation (Zatz *et al.*, 1998).

11.4.4 New mutation and mosaicism

At least 10–20% of all cases of FSHD are believed to result from *de novo* (new) mutation (Zatz *et al.*, 1995; Lunt, 1998; van der Maarel *et al.*, 2000). Proof of new mutation requires that DNA is available for study from both parents as well as from the affected index case, and that the specific D4Z4 deletion is found in the index case but not in either parent. Paternity must also be proven to be correct. In some cases of clinically apparent new mutation, this in fact occurs first in mitosis as a somatic mutation in one of the parents (Upadhyaya *et al.*, 1995; Köhler *et al.*, 1996). The parent is then a somatic (and germline) mosaic for the mutation, but may remain asymptomatic. The situation of somatic mosaicism is recognized on the electrophoretic gel of the parent's DNA as a band (DNA fragment) of reduced intensity (i.e. appearing fainter than the other bands). Mosaicism is confirmed if this manifests as an extra (5th) band on a pulsed-field gel which is also detecting the two normal 4q35 repeat arrays and the two 10q26 repeat arrays (van der Maarel *et al.*, 2000). It is interesting to speculate as to whether the nature of the D4Z4 repeats predisposes to replication errors at mitosis, or whether there are interference and gene conversion phenomena between the subtelomeric regions of chromosomes 4 and 10.

Overall, it is estimated that upwards of 40% of new mutations in FSHD arise in mitosis rather than meiosis, thereby giving rise to someone with somatic mosaicism (Upadhyaya *et al.*, 1995; Köhler *et al.*, 1996; Lunt *et al.*, 1998; van der Maarel *et al.*, 2000). Some of these cases only become recognized when they have affected offspring. In others, the mosaic mutation will give rise to symptoms, such that a proportion of molecularly proven new mutation cases will in fact be themselves mosaic.

Mosaicism can be:

– somatic alone: mutation occurs in mitosis but does not affect the germline
– gonosomal (somatic and germline): mutation occurs in a very early mitosis such that some of the germline cells also carry the mutation
– germline alone: mutation occurs in mitosis but only in the germ cell precursors which will undergo meiosis to yield the germ cells (egg or sperm).

Mosaicism recognized on DNA from peripheral blood leucocytes must be somatic mosaicism, whether alone or including the germline. The mosaic person's offspring will be at risk of inheriting FSHD if the germline is involved, but a wider family history would not be seen. If the mosaicism is only in the germline, there would not be any clinical effect on the individual involved and it would only be recognized if they had multiple affected offspring. Most mosaics recognized to date have been 'high level mosaics' suggesting that the mutation has occurred at one of the earliest cell divisions of the zygote, and also therefore involving the germline (van der Maarel *et al.*, 2000).

The effect of a mutation being in mosaic form is to confer a lesser clinical effect and severity than for a constitutional (full) mutation. Because of this, and due to an apparent higher mutation rate for larger deletions (see below), almost all recognized mosaics will have residual D4Z4 fragments of 1–4 repeats (10–20 kb *Eco*RI fragment). With the mosaicism, the distribution of affected muscle involvement may potentially also be

unusual, although a relatively even distribution throughout the body of mutant and normal cell lines may be expected if the mutation arises in an early zygotic division.

Typically, mosaicism in males tends to present with clinical features of FSHD, but is relatively milder than might be expected for the particular number of residual copies of D4Z4 (residual fragment length). Females who are mosaic tend to be recognized as the unaffected parent of a child with a relatively severe FSHD presentation (van der Maarel *et al.,* 2000). Quantitative DNA dosage studies (van der Maarel *et al.,* 2000) support this pattern for mosaics. These studies suggest that it requires a higher proportion of cells to be carrying the mutation for a female to manifest symptoms than for a male with a similar residual D4Z4 copy number. Therefore, within the limits of the range of residual fragment sizes seen in new mutations (see below), it is postulated that the proportion of mutant cells required to cause symptoms increases with increasing residual D4Z4 copy number. Although the finding in parents of *de novo* cases, that gonosomal mosaicism was observed much more commonly in mothers than in fathers suggested that this might be more common in females than in males (Upadhyaya *et al.,* 1995; Kohler *et al.,* 1996; Lunt *et al.,* 1998), the more recent findings above (van der Maarel *et al.,* 2000) now provide an explanation for this discrepancy. Thus, clinical expression appears to be associated with gender and fragment size, not only in cases with a constitutional mutation, but also in mosaics.

11.4.5 Comparison of clinical and genetic properties between new mutation and familial cases

Almost all recorded cases of proven *de novo* mutation (see above) in FSHD, whether as constitutional or somatic mutations, are associated with a residual number of D4Z4 repeats in the lower half of the affected range (1–5 repeats, or 10–20 kb *Eco*RI fragment size) (Lunt *et al.,* 1995a). This could either be due to an ascertainment bias, since the smaller the residual number of repeats, the greater the severity and the less likely are such cases to be familial, or an indication of a truly higher mutation rate for larger deletions. If mutation rates are equal and independent of deletion size, one would have expected to see as many apparent *de novo* cases as progenitors for subsequent typical dominant families, as are seen amongst more severe isolated cases. In practice, the parents of a milder new mutation case are likely to be older at the time of ascertainment than the parents of a more severe younger-onset new mutation case, and therefore may not be so readily available or willing to participate in a study. Hence it may be harder to prove new mutation in cases with higher residual D4Z4 copy number (larger residual fragment size). However, it is noteworthy that proven new mutation cases who have been reported as the progenitors of subsequent affected generations, have also had relatively few residual repeats (2–3 repeats) as would be typical for isolated new mutation cases (Lunt *et al.,* 1995a). These transmitted new mutation families also demonstrate the smallest residual fragment lengths seen in transmitted familial cases (see *Figure 11.1*). Indeed, the range of residual repeat number (fragment size) seen in new mutation cases, apart from in these two families, otherwise displayed little or no overlap with that seen in familial cases, as shown in *Figure 11.1* (from Lunt *et al.,* 1995a). It does seem most likely therefore that the new mutation rate is higher with respect to the smaller residual fragment lengths than to the larger ones.

11.4.6 Three categories of family presentation of FSHD (types I, II, III)

Conceptually, it may be helpful to be able to divide a combined clinical presentation and family structure for cases of FSHD into three different categories (types I, II, III). Broadly these would be (Lunt *et al.,* 1995b): type I—new mutation cases; type II—classical familial cases; type III—cases with larger residual fragment lengths and milder presentation.

Type I

These are new mutation cases with severe, early onset. Rarely are other family members affected, other than direct offspring. The residual *Eco*RI fragment size is usually 10–20 kb (1–4 repeats). If a parent is more mildly affected, this may indicate somatic mosaicism. If a sib is affected but not either parent, this will almost certainly be due to mosaicism in one parent, who would most often be the mother if neither has any clinical signs. Males may tend overall to be more severe/earlier onset than females.

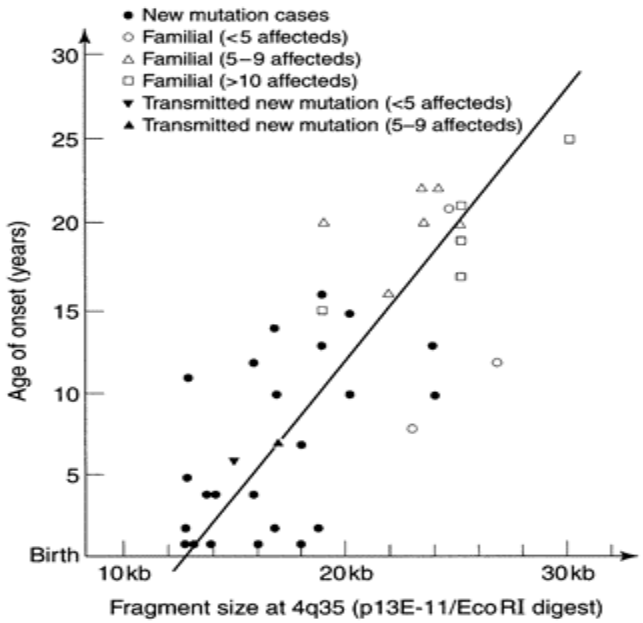

Figure 11.1 Correlation between age at onset and D4Z4 residual fragment size in FSHD for isolated cases and familial cases by size of family (redrawn from Lunt *et al.*, 1995a, Human Molecular Genetics, Oxford

University Press, by permission of
Oxford University Press.)

Type II

These are the typical classical familial cases, often with an extensive known family history of affected persons. Clinical severity will show quite wide variation, including occasional non-manifesting gene carriers, who would mostly be women. D4Z4 fragment size is often 20–32 kb (4–8 repeats). New mutation is rare.

Type III

These have a relatively mild presentation, typically with mostly shoulder girdle involvement. Some may have a scapulohumeral presentation with little or no facial weakness (Jardine *et al.*, 1994a, Butz *et al.*, 2003). Few other family members are usually known to be affected, but on molecular testing, many others, and particularly women, will be found to carry the same (large) D4Z4 fragment while remaining asymptomatic. *Eco*RI fragment size will typically be 32–41 kb (8–11 repeats). The average severity of known affected cases may appear to be little different from that in typical large families. However, countering this, there is likely to be a relatively large reservoir of clinically asymptomatic gene mutation carriers, who if identified and included would 'dilute' the apparent severity effect. In this group there is definite overlap with a control (normal) population, but studies on the telomeric A and B polymorphism have yet to be run in order to determine whether the overlapping controls do also have a type A telomeric allele, and therefore represent true overlap. If so, there would be a continuum between the normal population and the mild end of the spectrum for FSHD, with increasing likelihood of having clinical signs as the residual D4Z4 repeat number reduces below around 10 copies (40 kb).

It is helpful also to summarize these data according to fragment size *(Table 11.1)*.

11.4.7 BlnI-sensitive alleles and the type A and B polymorphism

The most recent genotype-phenotype correlation to be found is the apparent requirement for a particular polymorphism telomeric to the D4Z4 repeats on 4q35 to be of one allele type (type 'A') rather than the alternative (type 'B') in order for a D4Z4 repeat deletion to cause FSHD (Lemmers *et al.*, 2002). Some evidence already suggests that deletion on a background of a type 'B' polymorphism may have no clinical effect (van der Maarel *et al.*, 2003), although, speculatively, this does perhaps not yet preclude an alternative presentation not recognized as FSHD. Interestingly, at the homologous region at 10q26 only type A alleles have been observed; the type B allele appearing to be unique to 4q35. One might also speculate that the process of translocation or gene conversion whereby a *Bln*I-sensitive allele could be present on 4q35 and give rise to FSHD, or where a *Bln*I-resistant allele locates to 10q26, might mean that both the chromosome 10 and 4 involved would have to have an 'A-type' telomeric allele for this to be possible. If so, diagnostic confirmation or exclusion from molecular testing in these cases would remain problematic.

11.4.8 Emerging exceptions to the overall correlations

Parent sharing same fragment

It is difftcult to understand how an asymptomatic parent could share a very low copy number of D4Z4 repeats (a very short 4q35 DNA fragment) with a severely affected son or daughter, but personal observation indicates that this can occur (Lunt *et al.,* 1998). Speculative, but possible, explanations could include:

1. undetected gonosomal mosaicism in the parent, with a very low proportion of the affected allele in their muscle cells
2. shortened *Bln*I-resistant repeat array on chromosome 10 in the parent, but which may have predisposed to a same-sized D4Z4 array at 4q35 in the affected child, either by translocation or DNA sequence conversion
3. 4q35 telomere in the parent is of type B, but converts or recombines to give a type A telomere in the child.

Upper borderline-sized fragment

Some FSHD patients with larger fragment sizes (9–11 D4Z4 repeat copies) have still shown relatively severe presentations (Butz *et al.,* 2003). Some of these cases have an atypical presentation for FSHD, including some with proximal lower-limb onset, and which may therefore have an alternative diagnosis. However, in the recent series of patients with borderline fragment sizes of ≥8 repeats (Butz *et al.,* 2003), 7/24 typical FSHD patients were classed clinically as severely affected. This might suggest that the established inverse correlation of severity with residual D4Z4 repeat copy number might not hold for these higher repeat numbers.

Table 11.1 FSHD: Clinical aspects by residual number of 4q35 D4Z4 repeats (estimated from *Eco*RI digest fragment size with permission from: Deymeer F. (ed): Neuromuscular Diseases: From Basic Mecanisms to Clinical Managment. Monogr. Clin. Neurosci. Basel, Karger, 2000, vol 18, pp. 44–60)

*Eco*RI Fragment size (kb)	Residual no. of repeat units	FSHD category (see 11.4.6)	Typical family	Typical presentation	Median onset Age	% requiring wheel chair	Minimum and average age for wheel chair	Penetrance
10–14	1–2	Type I	(a) New mutation or sibs	Severe infantile onset	Infancy-2 years	> 50	8 years minimum 18 years	? Full, even mosaics

							median	
15–20	3–4		(b) New mutation or small family	Severe in adulthood	8–12 years	> 20	11 years minimum 35 years median	? Full, except mosaics
20–32	4–8	Type II	Large multiage neration family	Variable, mild to mod./severe	15–25 years	10–20	32 years minimum 40 years median	Male: 95% Female: ? 70–80%
33–41	8–11	Type III	(a) Often 'single' cases or 'small' families with non-penetrance/ cryptic carriers'	Very variable, usually mild, and usually as scapulo-humeral	20–40 years	< 10	Rarely: ? >40 years minimum ? >50 years median	Male: ? 80% or lower Female: ? 60% or lower

However, in practice, the discrepancy may simply reflect ascertainment of only single members in families where many others unknowingly carry the mutation but remain asymptomatic. The effect will be that only the most severe come to attention. The likelihood that a gene carrier in a family would be symptomatic may continue to fall according to higher residual repeat copy number for the family. Therefore, the higher the number of repeats, the greater will be the proportion of non-penetrant gene carriers in the family. In the large 'standard' families, typically with residual copy number of 5–7 repeats, it is easier to ascertain gene carriers to extend family studies, which may thereby include several minimally affected individuals. The severity of an index case with a larger residual repeat number from a different family, may therefore appear similar to the averaged severity in these larger families.

Over the coming years, as the molecular and cellular bases of the observed correlation are elucidated, we shall see whether the broad genotype-phenotype correlation holds even into the range of the borderline-length D4Z4 fragments which can also be seen in the normal population. If so, the effect of D4Z4 deletion on each individual muscle fibre can be considered as being influenced primarily by a probability determined by the residual D4Z4 copy number.

11.5 Fitting severity variation and correlation with molecular models

The overall inverse correlation between severity and residual D4Z4 copy number has now been corroborated by many different research groups (Lunt *et al.*, 1995a, 1995b; Zatz *et al.*, 1995; Tawil *et al.*, 1996; Padberg, 1998). Therefore any models constructed to explain the link between D4Z4 deletion and FSHD must also be able to account for the observed correlation. Supportive experimental evidence at the cellular level should

therefore also demonstrate differences in effect that are dependent on residual D4Z4 copy number.

11.5.1 Proposed models

Spreading heterochromatinization

Historically this was the first proposed model. It proposed that DNA is inactivated for a certain distance proximally from the telomere. If the inactivated region normally included the D4Z4 repeats, or if the repeats would normally act as a buffer to the spread of heterochromatinization, deletion of most of the repeats would result in a further proximal spread of the inactivated DNA, and might switch off genes more proximally in proportion to the length of DNA deleted (Winokur *et al.*, 1994). However, since haploinsufficiency of 4q35-qter region does not cause FSHD (Tupler *et al.*, 1996), this model is hard to sustain.

Subsequently, models have focused on transcriptional derepression mechanisms involving genes located more proximally (Gabellini *et al.*, 2003). These include the following.

Other physical effect of DNA on genes located more proximally

The D4Z4 repeats might provide a physical DNA structure (e.g. a coiled region with each 3.3 kb D4Z4 *Kpn*I unit forming each turn of the coil), which normally represses more proximal gene expression. Deletion of integral numbers of repeats would alter the DNA structure (e.g. by reducing the number of turns of the coil) in proportion to the number of repeats deleted, and hence allow proportional derepression of more proximal genes.

Direct effect of D4Z4 repeats

The D4Z4 repeats, or any RNA or protein product from them, may normally act directly as regulators for expression of other genes, or may act as targets for other regulatory elements (Ding *et al.*, 1998; Gabellini *et al.*, 2002, 2003). The effectiveness will be proportional to the number of copies of the repeat sequence still present. This model is one of transcriptional derepression (Gabellini *et al.*, 2003).

11.5.2 Transcriptional derepression

Several genes have been studied as the more proximal potential target genes including FRG1 (FSH-region gene 1), *TUB4Q* (tubulin ß-polypeptide 4q gene), *FRG2* (FSH-region gene 2) and *ANT1* (adenine nucleotide translocator-1 gene) (Gabellini *et al.*, 2002; Lemmers *et al.*, 2002). There is evidence to support transcriptional derepression of *FRG1*, *FRG2* and *ANT1* in FSHD, with the level of overexpression of these genes being inversely proportional to the distance proximally from the contracted D4Z4 repeats (Gabellini *et al.*, 2002). Furthermore, study of *FRG2* expression in muscle showed this to be expressed only in FSHD muscle and not in controls, and that the degree of *FRG2* expression correlated inversely with the residual number of D4Z4 repeat units (Gabellini

et al., 2002). It remains to be seen whether the observation on *FRG2* gene expression in particular proves to be a direct clue as to the pathogenesis of FSHD, or whether this represents a secondary effect in affected FSHD muscle, and of no greater significance than the altered expression of the many other genes which may be involved. Interestingly, in two unrelated FSHD families, *FRG2* was deleted on the disease chromosome, which suggests that *FRG2* is less likely to be a candidate gene for FSHD in these families (Lemmers *et al.,* 2003). Microarray expression studies have shown many of the genes dysregulated in FSHD are involved in myogenesis, cellular differentiation and cell cycle control (Winokur *et al.,* 2003).

Any molecular model must now also take account of the most recent genotype-phenotype correlative finding that FSHD only results when a D4Z4 deletion occurs on a chromosome 4 that has a 4qA-type polymorphism in the telomeric sequences, rather than a 4q B-type polymorphism (Lemmers *et al.,* 2002). If there is transcriptional derepression, the 4qA allele must in some way facilitate this, whereas the 4qB allele must protect against it (Lemmers *et al.,* 2002).

11.5.3 Understanding clinical variation in FSHD

One of the main challenges in understanding FSHD, and one which could potentially become of most importance therapeutically, is the wide range of clinical severity that can be observed within families between affected people of the same sex. This represents the variability of expression once the known variables of gender difference, residual D4Z4 copy number, and mosaicism are removed. There are no studies suggesting that the unaffected D4Z4 repeats or the 10q26 repeats have any role in this variation. Rather, although likely to be due predominantly to random variation and the overall genetic background, potentially more controllable factors might be the modifying effects of any more specifically interacting genes, and more general lifestyle factors. The study of differences in microexpression arrays in different FSHD patients, or linkage studies across large affected sibships may potentially be able to suggest candidates for any modifying genes, and hence perhaps lead eventually to therapeutic interventions.

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12.
Mosaicism and FSHD

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12.1 Introduction

The term mosaic was used in ancient times to refer to floors or windows with numerous little colourful pieces of stone or glass representing mostly religious or historical events. In a biological context, mosaicism may refer to two distinct phenomena. Mosaicism can refer to a viral disease of plants, resulting in light and dark areas in the leaves, which then often become shrivelled. An alternative definition, and one more appropriate for this chapter, is a condition in which an individual has two or more genetically distinct cell lines derived from a single zygote, but differing by virtue of the presence of a mutation or non-disjunction (Gelehrter *et al.*, 1998). The first paper on the existence of chromosomal mosaicism was published in 1959. Here, the phenotype of a case of Klinefelter syndrome was described, in a man with two distinct cell populations in his cultured bone marrow. One cell population contained 46 chromosomes with XX, whereas the other cell population contained 47 chromosomes with XXY (Ford *et al.*, 1959). Nowadays, it has become clear that mosaicism is an important factor that contributes to variability in phenotype, severity of disease and inheritance. Mosaicism has already been described for more than 30 different diseases, including both Mendelian and non-Mendelian disorders, e.g. haemophilia, Duchenne muscular dystrophy, Friedreich ataxia and FSHD (Gottlieb *et al.*, 2001).

12.2 Mosaicism explained

Mosaicism is a very common phenomenon in multicellular organisms. In humans, for example, female cells display random inactivation of the maternally or paternally derived X-chromosome, and so they exhibit mosaicism for the expression of specific genes on the X-chromosome. Moreover, as the number of cells in the adult human body (10^{14}) exceeds the mutation frequency of most genes, one must conclude that every individual must

exhibit some degree of genetic mosaicism. Thus, everyone probably carries mutations in any given gene in some proportion of their cells (Hall, 1988).

Mosaic cell populations can be the result of mutations in nuclear or mitochondrial DNA in postzygotic cells, epigenetic alterations of DNA, numerical or structural chromosomal abnormalities (such as missing or extra chromosomes) or of spontaneous reversion of inherited mutations. This may occur in any given cell and can be passed on to all daughter cells at any particular point in development or lifetime of an individual. From here on, any mutation described in this chapter will be referred to as a mosaicism-inducing event. The consequences of these mutations depend on the nature of the mutation, the cells in which they are expressed and their timing (Hall, 1988; Youssoufian and Pyeritz, 2002). When the mutation occurs in one of the first few cell divisions after fertilization, it yields a larger proportion of mutant cells in the total cell population than when the mutation occurs in a later developmental stage (Pearson, 2002).

One of the most important issues in genetic counselling is whether a mutation is likely to be transmitted to the next generation. This depends on the nature of the mutation and more importantly, when and where it occurs. A postzygotic mutation can be passed on to the offspring only if it is present in cells that give rise to germ cells. Therefore, a distinction is made between germline mosaicism and somatic mosaicism. In the latter form, the absence of the mutation in germ cells will prevent transmission to progeny. When mosaicism arises during an early stage in embryogenesis, the same mutation can coexist in both somatic and germ cells within the same individual. This is dependent on the specific cell (type) affected and the developmental timing of the mosaicism-inducing event. A mutation will be passed on through the germline but will be absent from somatic cells only if it occurs in cells already committed to germline formation (Hall, 1988; Youssoufian and Pyeritz, 2002).

Cancer pathology is probably the best known example of mosaicism (Bernards and Gusella, 1994; Evans *et al.,* 1998). Nearly all cancers are monoclonal in origin, and a critical mutation event has (or multiple mutations have) transformed this single cell into a cancer cell with uncontrolled proliferation leading to the formation of a neoplasm. When a mosaic cell population arises due to a mutation in a tumour suppressor gene, this mutation represents the ‘first hit’ towards the development of cancer in these cells. Cells carrying such a mutation are predisposed to malignant formation once a second somatic mutation has inactivated the remaining normal copy of the gene. In the hereditary cancer retinoblastoma, for example, somatic and germline mosaicism for the initial mutation in the retinoblastoma *(RB1)* gene is well documented. Not all mosaic individuals have or will develop retinoblastoma (Sippel *et al.,* 1998). In a severe autosomal dominant disorder that predisposes to multiple benign tumours of the nervous system, neurofibromatosis type 2 (NF2), mosaicism has been observed in as much as 25% of NF2 founders and is proposed to account for the high number of mutations observed (Kluwe *et al.,* 2003). Germline and somatic mosaicism associated with human cancer are also observed in, for example, neurofibromatosis type 1, testicular tumour development and von Hippel-Lindau disease (Lazaro *et al.,* 1994; Murgia *et al.,* 1999; Bianchi *et al.,* 2002) and is now regarded as a major contributor to the development of the pathogenesis of cancer.

12.3 Somatic mosaicism

Somatic mosaicism is depicted in *Figure 12.1*, and appears when a mutation arises after the commitment to germ cell formation. This has been observed in several diseases representing different systems in the human body, such as skeletal disorders (e.g. Marfan syndrome), skin disorders (e.g. incontinentia pigmenti) and metabolic disorders (e.g. adenosine deaminase deficiency) (Youssoufian and Pyeritz, 2002).

The phenotypic consequences of a somatic mutation depend on various factors such as the cell type(s), tissues and/or organs in which the mutation is expressed. A mutation may be tolerated in some cells, whilst being lethal in others or instead having a selective advantage as observed in malignancies. When a gene is necessary for normal cell function in a certain tissue, cells with a mutated gene may then be selected against by non-mutated cells and hence induce mosaicism, whilst the same mutation is tolerated in other tissues (Hall, 1988). Therefore, phenotypic variability in expression levels can, besides alterations in DNA sequences in specific genes or mutations in modifying genes, also be influenced by mosaicism. The timing of somatic mutations will then be important, as an early mutation is likely to have a greater effect on the expressed phenotype (Gottlieb *et al.*, 2001).

As an example, some males with the X-linked dominant disease Rett syndrome survive due to somatic mosaicism (Topcu *et al.*, 2002). In such a condition, normal

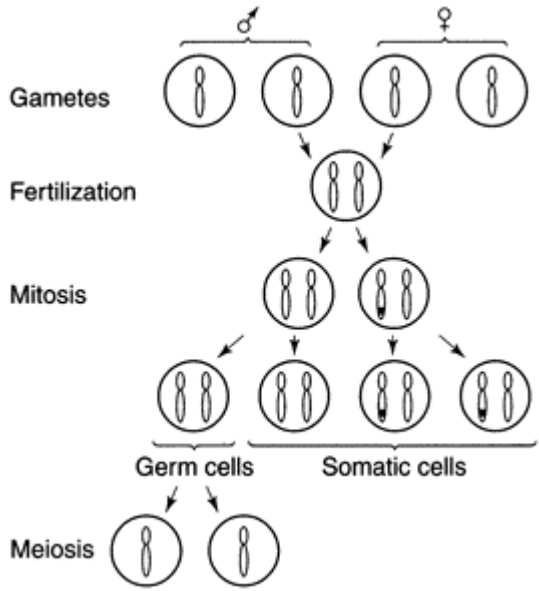

Figure 12.1 Schematic overview of development and inheritance risk for somatic mosaicism. The black bar in the chromosome represents the

disease-causing mutation, that will be visible in cells due to clonal expansion of somatic cells carrying this disease-causing mutation. In this scenario, the offspring is not at risk since the mutation is not present in gonadal tissue.

cells may arise that outgrow the mutant cell population. The healthy cells will spread throughout the developing individual and compensate for the mutant cells, allowing survival of the individual who would otherwise have died during development. Although it is not yet understood why these mutations occur before fertilization or as a postzygotic event, the presence of an X-linked disease in a male is rare and is therefore an important diagnostic indicator of the occurrence of somatic mosaicism in that individual (Ozcelik, 2002).

Numerical or structural abnormalities of chromosomes are common causes of somatic mosaicism. Chromosomal mosaicism, such as a deviation in the number of chromosomes (aneuploidy), is observed in several disorders. The identification of these forms of somatic mosaicism can be complicated by the tissue-restricted presence of the marker chromosomes. This is, for example, shown in individuals with the Pallister-Killian syndrome, a severe congenital malformation syndrome that includes features like mental retardation and postnatal growth deficiency, which is caused by a tetrasomy of the short arm of chromosome 12. This marker chromosome is present in most bone marrow mononuclear cells, but detectable in only a small fraction of peripheral blood mononuclear cells. So, the genetic identification of the tetrasomy will here be hampered by the restriction of mosaicism to a specific tissue or cell type lineage (Youssoufian and Pyeritz, 2002).

12.4 Germline mosaicism

When in a family with no history of disease, two or more children manifest a phenotype of an autosomal dominant or X-linked disorder, germline mosaicism in a substantial number of germ cells in one of the parents could be the underlying mechanism. This is an important mechanism for disease and has been demonstrated in disorders like osteogenesis imperfecta, haemophilia A and Duchenne muscular dystrophy (Hall, 1988; Cohn *et al.*, 1990; van Essen *et al.*, 1992; Becker *et al.*, 1996; Youssoufian and Pyeritz, 2002). In this type of mosaicism, the mutation can occur (1) in a germ cell that continues to divide or (2) very early in development, before the cell fate determination to germinal or somatic cells, and will thus be present in both somatic and germ cells (see *Figure 12.2*). Factors such as the gene or locus involved, tissue specificity and the degree of mosaicism will eventually determine if a carrier may be asymptomatic or present various characteristics of a disease (Zlotogora, 1998). The exact risk of passing on a disease-associated mutation is variable in this case, but can be as high as 50%.

A classification in two different groups of germline mosaicism-associated disorders has been proposed by Zlotogora (1998). The first group contains parents in whom mosaicism is suspected because there is transmission of the mutation to two or more children while they themselves have very mild symptoms or no disease characteristics at all. In the second group, parents present clinical symptoms of a disease caused by the expression of somatic mosaicism and have at least one child who has received the mutation and is clinically affected, indicative of germline mosaicism. The mutation present in both somatic and germ cells confirms that the mutation most likely occurred very early on in embryonic development (visualized in *Figure 12.2*).

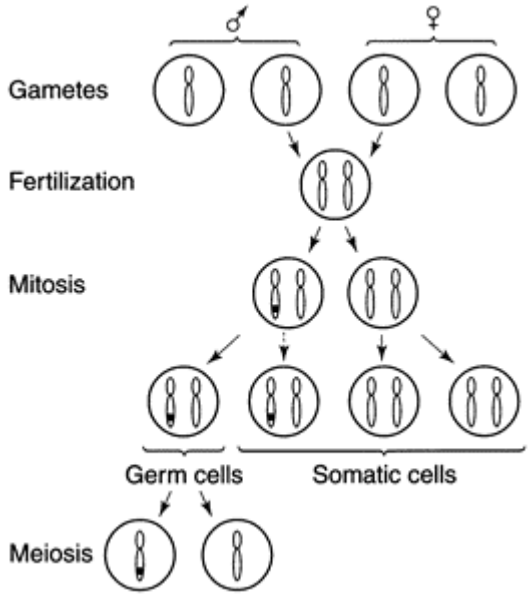

Figure 12.2 Schematic overview of development and inheritance risk for germline mosaicism only, or both somatic and germline mosaicism. The black bar in the chromosome represents the disease-inducing mutation which, depending on the developmental stage in which the disease-inducing mutation occurs, spreads throughout somatic and germ cells or germ cells alone (possibility indicated by dotted arrow). Depending on the timing, the mutation can already

be identified in clinically unaffected parents of non-mosaic patients. The inheritance risk for future offspring depends on the proportion of germ cells that contain the mutation. The maximum risk will be 50%, as shown for other diseases (Zlotogora, 1998).

12.5 Somatic and germline mosaicism in FSHD

In 1993, Weiffenbach *et al.* (1993) published difficulties in mapping the FSHD gene to 4q35 owing to recombination events. In this paper, they also briefly mentioned two families with germline mosaicism. Examination of the parents and offspring in these two families revealed clinically confirmed FSHD in the children, but no signs of muscle weakness in the face or shoulder girdle of both couples of parents. In both families, DNA analysis on blood lymphocytes confirmed a short fragment in all patients, but no short bands present in either parent. This suggests that only some or all germ cells of the father or mother contain the mutation and no somatic cells (at least no lymphocytes) are affected. This mutation probably occurred in a germ cell that continued to divide. In the same year, another publication by Griggs *et al.* (1993) describing new mutations in sporadic cases, mentioned the same observation in one of their seven families studied (Griggs *et al.*, 1993).

The first complete survey dedicated to studying germline mosaicism in FSHD was published in 1995 (Upadhyaya *et al.*, 1995). This article reported three sporadic FSHD families in whom the affected proband had inherited a potential rearranged D4Z4 fragment from one of the parents and was severely affected in all three cases. Analysis of DNA isolated from blood lymphocytes showed in all three families that the short D4Z4 fragment in the child was also visible in the mother, but the fragment was less intense after hybridization with probe p13E-11. This indicated that these three mothers transmitted the disease allele to their children via the germ cells. Since only a faint FSHD fragment was seen in the lymphocytes of these mothers, a small percentage of their somatic cells must be mosaic for the short D4Z4 fragment. As the mutation is present in both gametes and somatic cells (here only lymphocytes are analyzed), the mutation must have occurred before the differentiation of cells into endodermal, ectodermal and mesodermal cell lineages (see also *Figure 12.2*). Parents of apparently isolated FSHD cases may have insufficient expression of the mutation in different tissues due to mosaicism and therefore show no, or only a very mild, FSHD phenotype. This may explain the early observation made by Brooke (1977) of the unusual manifestations of severe infantile FSHD with a minimally affected parent. With these new insights, germline mosaicism could explain the occurrence of several affected FSHD offspring of unaffected parents and close the debate regarding possible autosomal recessive inheritance of FSHD (Upadhyaya *et al.*, 1995). In addition, the occurrence of mosaicism may, at least partially, explain the anticipation noted in some FSHD families.

Subsequently, mosaicism was reported by other groups. All available literature data are summarized in *Tables 12.1a* (patients) and *12.1b* (parents) (Griggs *et al.*, 1993; Weiffenbach *et al.*, 1993; Padberg *et al.*, 1995; Upadhyaya *et al.*, 1995; Zatz *et al.*, 1995, 1998; Bakker *et al.*, 1996; Kohler *et al.*, 1996; Hsu *et al.*, 1997; Lunt, 1998; Roques *et al.*, 1998; Galluzzi *et al.*, 1999; van der Maarel *et al.*, 2000). A very large group of parental somatic and germline mosaics was published by Köhler *et al.* analysing both parents of affected offspring. They observed 26% mosaicism in a total of 42 families grouped in families with autosomal dominant inheritance, families with one sporadic patient and families with clinically very mild or unaffected parents, no FSHD family history and multiple affected offspring (Kohler *et al.*, 1996).

van der Maarel *et al.* (2000) showed in a survey of 35 *de novo* FSHD families that somatic mosaicism was present in 40% of cases (14/35), either in the patient or an asymptomatic parent. Using pulsed-field gel electrophoresis (PFGE), they scored

Table 12.1a Summary of all available data, only 11 patients have been reported to be mosaic for a chromosome 4 allele associated with FSHD in a total of 78 mosaic individuals. However, one must take into account here, that most research laboratories tested the individuals with conventional gel electrophoresis, while mosaic alleles are more easily visualized with PFGE. Two patients transmitted the mosaic allele to their offspring and are therefore also mosaic in their germ cells. From the other nine patients gonadal tissue is not analyzed and transmission of the mosaic allele has not yet been observed, because these individuals did not have children at the moment of lymphocyte testing. The asterisk (*) indicates that the total number of families tested in each study included both inherited and *de novo* mutations.

	Family (n)		Germline (n)		Somatic (n)	
Reference	mosaic	total*	male	female	male	female
G Galluzzi *et al.;* 1999	7	145	–	2	–	2
SM van der Maarel *et al.;* 2000	14	35	–	–	7	2
Total	**21**	**180**	**0**	**2**	**7**	**4**

Table 12.1b Several research groups reported mosaicism in one of the parents. From the 712 families tested, 67 parents were mosaic: nine FSHD individuals with multiple affected offspring were only mosaic in their germ cells, because no short fragment was observed in their lymphocytes. In 58 individuals mosaicism was observed in both somatic and germ cells. Somatic mosaicism was always analyzed in lymphocyte DNA. Germline mosaicism, however, was always suggested by multiple affected offsprings, but in none of the cases was gonadal tissue tested to confirm the presence of the mosaic allele. The asterisk (*) indicates that the total number of families tested in each study included both inherited and *de novo* mutations. nr: gender not reported. a: Here only DNA from two families was analyzed, no short fragment was observed in lymphocytes. b: Data collected on the 44th ENMC international workshop; this is a a summary from other articles and includes results which have been previously presented. Two groups summarized in this report (15/128 and 87/128) may also still include mosaic individuals, only the 26/128 are established mosaic cases. c: In this study, one female was already reported in 1995 by this author and is not included here. d: only from one mosaic parent the gender is reported.

	Family (n)		Germline (n)			Somatic (n)		
Reference	mosaic	total*	male	female	total	male	female	total
B Weiffenback *et al.;* 1993	2	24	nr	nr	2	–	–	0
RC Griggs *et al.;* 1993	1	7	nr	nr	1	–	–	0
M Upadhyaya *et al.;* 1 995	3	34	–	3	3	–	3	3
GW Padberg *et al.;* 1995	5[a]	139	nr	nr	5	–	–	0
M Zatz *et al.;* 1995	1	34	–	1	1	–	1	1
J Köhler *et al.;* 1996	11	42	2	9	11	2	9	11

E Bakker *et al.;* 1996	3	58	3	–	3	3	–	3
Y-D Hus *et al.;* 1997	1	13	nr	nr	1	–	–	0
PW Lunt; 1998	26[b]	128	5	21	26	5	21	26
I Roques *et al.;* 1 998	1	1	1	–	1	1	–	1
M Zatz *et al.;* 1998	3[c]	52	1	2	3	1	2	3
G Galluzzi *et al.;* 1999	7[d]	145	nr	1	5	nr	1	5
SM va der Maarel *et al.;* 2000	14	35	2	3	5	2	3	5
Total	**78**	**712**	**14**	**40**	**67**	**14**	**40**	**58**

mosaicism as a fifth fragment hybridizing with probe p13E-11. In five families (14%), a parent was mosaic in his or her lymphocytes and passed the mutant allele that was rearranged as an FSHD-sized allele, to their child. This indicates that these parents are probably also mosaic in their germ cells. However, gonadal tissue would have to be tested to confirm this observation.

In nine patients (26%), a fifth fragment was also observed in lymphocyte analysis, which was not observed before in the family. Strikingly, mosaicism for a short FSHD allele was more often seen in male than in female patients (7 vs. 2). The proportion of cells carrying the short D4Z4 fragment in the FSHD patients ranged from 20% to 95%. Comparing male and female FSHD patients revealed a higher proportion of the deleted allele in female patients (average percentage of 80% in females vs. 31% in males). If this is combined with the female excess among unaffected parents (n=14 vs. n=40; reported cases in *Table 12.1b*), a higher clinical tolerance for mosaic disease alleles in females is proposed. Consistent with this finding, males are more severely affected than females (Padberg, 1982; Lunt *et al.,* 1989; Padberg *et al.,* 1995; Zatz *et al.,* 1998).

With this method they were also able to determine repeat array constitutions in mosaic individuals. In each individual, chromosome 4 and 10 repeat arrays are analysed by PFGE. The chromosomal origin of all fragments can be determined after hybridization taking advantage of a *Bln*I restriction site that allows discrimination of chromosome 4 and 10 arrays (see Chapters 7 and 15 for a more detailed explanation). Most individuals analyzed so far display a standard pattern of 4-type arrays on chromosome 4 and 10-type arrays on chromosome 10. Some 20% of individuals have translocated repeat arrays, which results in the presence of 10-type arrays on chromosome 4 and 4-type arrays on chromosome 10 (van Deutekom *et al.,* 1996; van Overveld *et al.,* 2000). However, analysis of the 13 mosaic individuals in whom all alleles could be scored yielded an unexpected result. In six mosaic cases, one or more 4-type arrays on chromosome 10 (46%) were observed. In the Dutch population, this type of repeat array constitution is present in only 10% of individuals. No mosaic individual had the reverse constitution (10-type arrays on chromosome 4) which is present in 10% of controls (van Deutekom *et al.,* 1996; van Overveld *et al.,* 2000). Thus, the presence of supernumerary identical, but not homologous D4Z4 repeat array configurations on chromosome 10 may influence the risk of development of FSHD.

Summarizing all cases reported so far, it becomes clear that, when a short D4Z4 fragment emerges in a pedigree and is passed on to subsequent generations, the

phenotype of the first individual carrying a short D4Z4 fragment is not relevant. The carrier of the initial short D4Z4 fragment causing the disease can be fully affected, but also an asymptomatic mosaic individual. Furthermore, according to all published data, most of the mosaic parents analyzed are female (n=14 vs. n=40; reported cases in *Table 12.1b*), putting mothers at a higher risk of being a carrier for a mosaic FSHD allele. Therefore, the observed germline mosaicism in families thought to represent an apparent new mutation with a low risk will now increase the recurrence risk of FSHD in future offspring and should thus be closely analyzed.

12.6 Implications for genetic counselling and research

Since molecular analysis is usually restricted to the analysis of DNA from blood lymphocytes, (a low level of) mosaicism in these individuals may go unnoticed. Since the degree of mosaicism may differ between tissues or may even be restricted to a specific tissue alone, the estimated frequency of mosaicism will be underestimated. The examination of several tissues and cell types is therefore necessary to increase our ability to detect mosaicism and to gain more insight into mosaicism and its consequences for the FSHD phenotype.

Furthermore, to obtain a complete visualization of all alleles, which is necessary to obtain a full understanding of mosaicism in FSHD, PFGE is recommended. This technique allows the separation of large fragments of DNA, which cannot be resolved by conventional gel electrophoresis. Applying PFGE, all genomic regions of interest can be analyzed and mosaicism can easily be scored as an extra band (or bands) when the mosaic fragment is larger than 20 kb.

In theory, three different types of FSHD mosaicism can be expected with different inheritance risks. Individuals carrying mosaicism for the short D4Z4 frag-ment only in somatic cells can present clinical characteristics of FSHD, depending on the percentages of mosaic cells in different tissues (type I, *Figure 12.1*). This type of mosaicism cannot be inherited from a mosaic individual, as the short D4Z4 fragment is only present in somatic cells. In this case, there is no elevated risk for the children to develop FSHD. On the other hand, individuals with only germline mosaicism will have no clinical symptoms of FSHD themselves, show no short fragment by analyzing lymphocyte DNA or any other somatic tissue, but will pass on a short D4Z4 fragment to their offspring (type II, *Figure 12.2*). One should suspect this form of mosaicism when healthy parents with no family history of FSHD have multiple affected offspring. This is, however, also possible when one of the parents is mosaic in both somatic and germ cells (type III, *Figure 12.2*) and shows no or very mild FSHD characteristics. Here, a short D4Z4 fragment will be detected in somatic tissue of the parent, for example in lymphocytes, at a reduced dosage. Both type II and type III mosaicism will result in elevated, but still variable recurrence risks for the offspring. It should be noted that distinction between types I and III will only be possible when FSHD analysis is performed in gonadal tissues. Furthermore, mosaicism in a parent (type II or III) shifts the *de novo* mutation in these families to the mosaic parent, resulting in an elevated risk of inheriting FSHD from a parent. Also, mosaicism in one of the minimally affected parents might in some cases of apparent *de novo* families provide an explanation for the clinical anticipation sometimes observed.

To focus on the significance of accurate genetic counselling, insight into the mutation rate for a disease is very important. In a recent study, the subtelomeric D4Z4 repeat array configuration of human chromosomes 4q and 10q was analyzed by PFGE in 208 unrelated healthy individuals (van Overveld *et al.*, 2000). Somatic mosaicism for one of the alleles was observed in as much as 3% of individuals for both 4-type and 10-type alleles, seen as a fifth repeat array fragment after hybridization with probe p13E-11. This high mutation frequency indicates dynamic behaviour for these two regions in the human genome and will therefore be important for risk assessment. However, mosaicism should not be confused with *de novo* mutations that are inherited through one of the parents. Of all cases investigated, FSHD has a relatively high proportion of new mutations of between 10% and 30% (Padberg, 1982; Zatz *et al.*, 1995, 1998; Padberg *et al.*, 1995; Tawil *et al.*, 1996; Lunt, 1998). To provide correct information in genetic counselling, one must attempt to determine if mosaicism is present and then distinguish mosaicism in somatic and germ cells, because risks and consequences for the progeny differ widely.

Interestingly, in all studies a female predominance of mosaic parents was reported. Since asymptomatic mosaic female parents may have as much as 40% of somatic cells carrying a short D4Z4 fragment, this suggests a higher clinical tolerance for the mutation in females. So, if an FSHD patient is born in a family without any history of the disease, inheritance via a mosaic parent rather than a *de novo* mutation in the patient himself or herself cannot be excluded. A thorough analysis of somatic and gonadal tissue if possible is recommended to give the most accurate advice regarding any risk for future pregnancies.

12.7 Summary

Mosaicism arises when an error disrupts the correct number of chromosomes segregating to each cell during cell division or creates a mutation in a single gene or locus (Pearson, 2002). The range of diseases that have been associated with mosaicism is very broad and includes almost all processes in the human body. Observations of mosaicism in all its forms thus have important clinical implications. Mostly, the effect of a mosaic population is masked, but can also result in major phenotypic expression and disease. All events that induce mosaicism can influence the phenotype in a negative or positive manner.

All studies on mosaicism in FSHD indicate a wide range of phenotypic expression of the two genetically distinct cell populations for a single chromosome 4 with one of the cell populations carrying a short D4Z4 fragment. Clinical effects of mosaicism can be very mild in adults, but parents could be at risk to pass on the condition to their children if their germ cells contain the mutated allele. The possibility of a parent being mosaic must therefore be taken into account when genetic testing is requested.

The mechanism that leads to the observed cell populations with five alleles is termed gene conversion, a non-reciprocal recombination process that results in an alteration of a particular sequence to a homologous sequence during meiosis or mitosis *(Figure 12.3a)* (Batzer and Deininger, 2002). In other words, when one allele is damaged due to a double strand break (for example in or near D4Z4 repeats on chromosome 4 or 10), the sequence of the homologous chromosome will be used as a template to repair the damage. This copying of the chromosome sequence can start on any arbitrary, but homologous point,

resulting in expanded or contracted chromosome sizes. In the case of mosaicism for D4Z4 rearrangements, this process will eventually lead to a mosaic cell population with three alleles present for chromosome 4 or 10.

The plasticity of the 4q35 subtelomeric region associated with FSHD has recently been emphasized at the 7th Annual Meeting of the World Muscle Society. Lemmers *et al.* (2002) presented three FSHD patients with an even more complex rearrangement resulting in three genetically distinct cell populations. In two patients, the original allele was shortened into two smaller alleles, of which one was FSHD-sized and causing disease. In the third individual, the original allele was mutated to a small FSHD-sized allele, but also expanded to an allele larger that the original one. These observations suggest that, alongside gene conversion, unequal recombination events, with resulting deletion and duplication, may also contribute to new mosaic cell populations. This mechanism is depicted in *Figure 12.3b*. The frequent observation of somatic mosaicism in FSHD may allow researchers to generate genetically identical cell lines that differ only in the size of the D4Z4 repeat array. These cell lines may eventually provide new insights in the pathogenesis of FSHD.

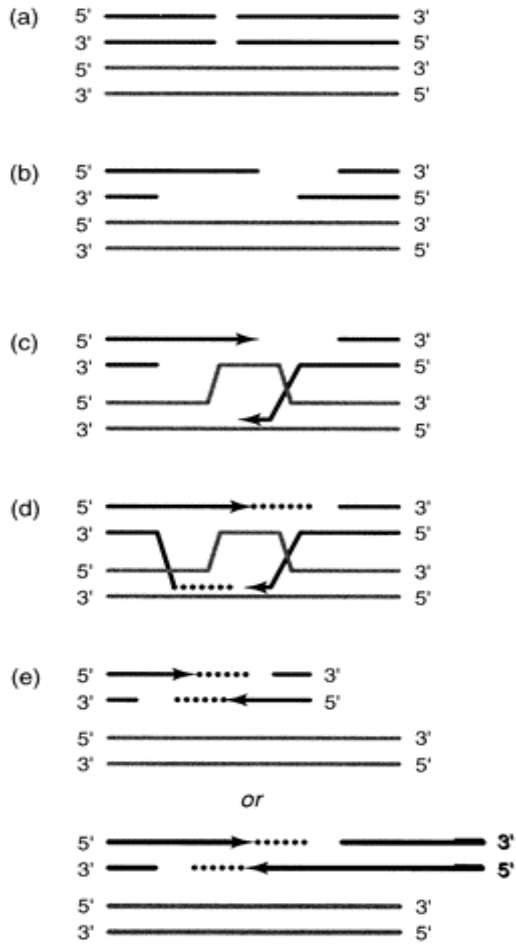

Figure 12.3a Visualization of gene conversion as a consequence of the process of unequal recombination. Homologous chromosomes involved are represented as black and grey lines. Gene conversion is the non-reciprocal transfer of genetic information, i.e. for the repair of the double strand break. (a) a homologous grey chromosome (or sister chromatid; not visualized) donates the genetic information to repair the break. After 5′ to 3′ resection

(b), the black chromosome uses the invaded segment from the donor (grey, c and d) as a template to fill in sequence lost due to the double strand break. This will result in a contraction or an expansion of the damaged black chromosome. The grey donor chromosome remains unchanged (e).

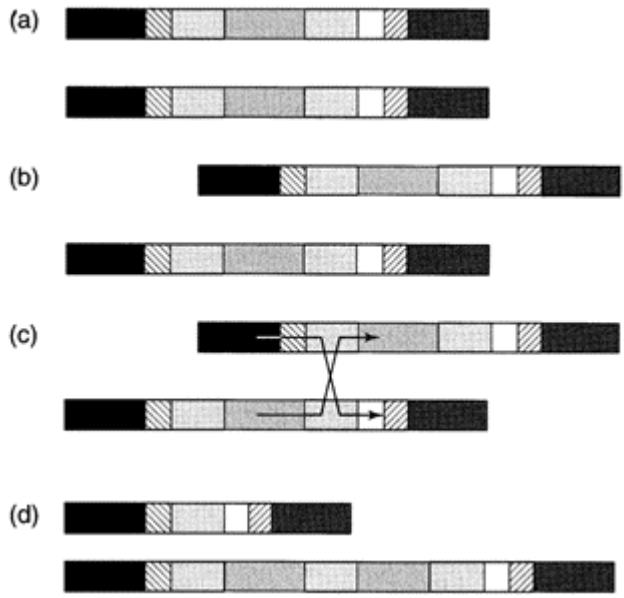

Figure 12.3b Visualization of an unequal crossover. Homologous chromosomes involved are represented as blocks with various patterns (a). Due to significant homology between sequences, a homologous chromosome may not align perfectly with its corresponding region on another chromosome during meiosis (b). A single strand break in both chromosomes will result in an unequal crossover and the cleaved strands will join with homologous strands (grey

lines in c). According to the Holliday model, the results of this unequal crossover are two chromosomes with different lengths due to the exchange of sequences (d).

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13.
Retinal vascular abnormalities in FSHD: a therapeutic message; clues to pathogenesis?

Robin B.Fitzsimons

13.1 Introduction

Facioscapulohumeral muscular dystrophy is an autosomal dominant myopathy characterized by marked asymmetry, patchy inflammation, capricious progression—and an idiosyncratic distribution of muscle weakness which in its classical presentation is simulated by few, if any, other disorders of muscle.

'Coats' disease' is an asymmetrical (indeed, generally unilateral) exudative retinopathy consequent upon an underlying telangiectasis of small retinal blood vessels (Coats, 1908). This telangiectasis is usually 'patchy' and often associated with inflammation. There is an overwhelming male predominance. When the disease is clinically bilateral, secondary to other disorders, or occurs in females, the term 'Coats syndrome' is sometimes used to avoid definitional problems, but as the condition is probably heterogeneous, such semantic 'splitting' may not be justifiable. The perennially predictable semantic 'lumpers versus splitters' debates, based on strict unilaterality and male-only occurrence, are unlikely to be resolved until the underlying pathogeneses of Coats' disease(s) are determined. Nevertheless, fluorescein angiography has shown that pathological bilaterality of Coats' disease is more common than clinical bilaterality, and that clinical bilaterality is sufficiently common as to warrant careful ophthalmic surveillance of the unaffected eye which might later need prophylactic treatment.

The primary abnormality in Coats' disease is thought to affect capillary endothelium (Tripathi and Ashton, 1971). Affected retinal blood vessels are wider and sparser than normal, and there are areas of capillary dropout in the retinal periphery. Microaneurysms are common, and there may be small retinal haemorrhages. When exudates from abnormally leaky vessels track to the more capacious posterior pole, massive exudative retinal detachment, with secondary glaucoma, may ensue, which in extreme forms simulates retinoblastoma and leads to enucleation of the eye.

There is now known to be a general assocation between the retinal telangiectasis which underlies Coats' disease (or syndrome) and FSHD (Fitzsimons *et al.*, 1987; Padberg *et al.*, 1995). Such telangiectasis in FSHD is mostly asymptomatic, but in a small minority of cases the effects on vision are devastating—unless timely treatment is instituted.

There is a number of recent reviews of Coats' disease, its presentation and treatment with laser photocoagulation (Campbell, 1976; Tarkkanen and Laatikainen, 1983; Char, 2000, Cahill *et al.*, 2001; O'Keefe *et al.*, 2001; Shields *et al.*, 2001a, 2001b; Alesandridou and Stavrou, 2002; Shields and Shields, 2002). These are directly relevant to patients with clinical retinal complications of FSHD.

The treatability of the rare clinical retinal disease of FSHD contrasts with the lack of specific available treatment for the universal myopathy. However, it places a premium on timely identification and treatment when sight-threatening retinal exudation occurs.

There is also an association between FSHD and hearing impairment (reviewed in Padberg *et al.*, 1995). Clinical deafness with symptomatic retinal disease is relatively commonly associated with the severe infantile form of FSHD, but is also present in milder forms of the condition.

This chapter will develop two themes. The first will concentrate upon the clear therapeutic implications of the FSHD/retinal telangiectasis association. The second is more speculative, and looks for clues to the pathogenesis of FSHD in the light of the known associations between on the one hand Coats' disease and FSHD, and on the other hand Coats' disease and other conditions such as retinal 'Norrie disease'. Do the known gene abnormalities in such other conditions shed light on possible genetic abnormalities in FSHD?

13.2 Retinal disease and FSHD

13.2.1 Background and therapeutics

In 1968, Small documented Coats' disease and hearing impairment in three siblings affected by a form of muscular dystrophy—which clearly had the characteristics of FSHD. 'Mental retardation' was also described. However, such 'retardation' might at least in part have been a perceived unresponsiveness related to profound hearing loss with facial weakness. Catastrophic imitation of mental retardation is a very real occurrence in infantile-onset FSHD consequent upon this unfortunate clinical combination of severe deafness and a lack of facial expression—which causes seeming indifference to the external world.

Then in 1982, Taylor *et al.* reported two cases of Coats' disease with hearing impairment in FSHD, and Wulff *et al.* documented a further case. There was nevertheless no suggestion of a general association between these conditions and FSHD.

In 1985, Gurwin *et al.* reported a family with FSHD in which the propositus had severe infantile-onset disease and a comparably severe hearing loss which had, in combination, led to an underestimation of her mental capacity. There were no clinical visual problems. However, her teenage sister, with relatively mild FSHD muscle disease, had had unilateral central visual loss in one eye since infancy because of a macular scar which had earlier been diagnosed as due to toxoplasmosis infection. Because of the earlier case reports of Coats' Disease with FSHD and the fact that Coats' Disease is known to have a vascular aetiology, the question arose as to whether the scar might rather have a vascular aetiology and in fact represent a systemic association of FSHD. I also asked whether her three visually unaffected relatives known to have FSHD might have

had retinal vascular abnormalities. Subsequent fluorescein angiography confirmed retinal telangiectasis in all four individuals, and clearly demonstrated that this telangiectatic disease had caused the macular scar and the associated permanent loss of central vision in one eye. The macular scar was anything but a 'sporadic' phenomenon.

That small study also raised the question of whether or not such underlying asymptomatic vascular phenomena might be a more general association of FSHD, or whether hearing loss and retinal disease merely defined a tiny subgroup of FSHD. Indeed, the first hypothesis proved to be correct in a much larger fluorescein angiography study of FSHD patients undertaken at Moorfields Eye Hospital, London. About two-thirds of the 75 affected individuals studied had demonstrable retinal vascular abnormalities on *peripheral* fluorescein angiography, even though in most cases this was not evident clinically, even on indirect ophthalmoscopy. Very few had evidence of clinically significant exudates or haemorrhages, even though when these were present they were amenable to aggressive laser photocoagulation therapy (Fitzsimons *et al.*, 1987). The general association between FSHD and retinal abnormalities was subsequently confirmed by Padberg *et al.* (1995), who demonstrated retinal vascular abnormalities in the majority of patients they studied with fluorescein angiography.

These general studies provided no reason to believe that the retinal changes defined any subset of FSHD. Such changes were seen in at least some members of all the larger families studied by Fitzsimons *et al.* (1987). However, often the changes were subtle and very focal, and could easily have been missed. In some cases, the findings were equivocal. Nevertheless, it is now apparent that whereas sight-threatening illness can present at any age or degree of severity of FSHD, it is more likely to occur, and to occur early, in the severe early-onset form of FSHD. Indeed it may antedate the onset of muscle disease.

Since then, there have been a number of reports of FSHD with clinical Coats' disease (Desai and Sabates, 1990; Pauleikhoff *et al.*, 1992; Bindoff *et al.*, 2001) and substantially greater numbers of which I am aware (particularly in the United States) that are as yet unreported. Some of these have required treatment and have come to ophthalmic attention through the screening of infants with FSHD or at genetic risk of FSHD.

Fluorescein angiography is not clinically warranted in FSHD patients unless sight-threatening abnormalities are seen on indirect ophthalmoscopy by a retinal ophthalmologist. Nevertheless, when performed as a research procedure or as an assessment tool in patients who by other criteria may require retinal treatment, the technical quality of the angiogram is likely to be crucial. In less overt cases of retinal disease, the most prominent abnormalities seen on angiography are likely to be at the retinal periphery. Peripheral photographs are not always included in standard fluorescein angiograms, and the angiographer will require considerable technical expertise to avoid 'camera astigmatism' when photographing the periphery. From a practical perspective it is important to alert the angiographer to photograph the periphery (particularly the temporal periphery) as well as the posterior pole. It is the posterior pole where the sight-threatening damage occurs—after exudates originating in the periphery have tracked to the macular area, and caused exudation or later scarring. In extreme cases this causes a massive 'whiteout' simulating retinoblastoma. Indeed, a 'retinoblastoma' with FSHD (Emery and Rimoin, 1983) was later shown to be Coats' disease.

Since sight-threatening exudates occur particularly commonly in young children, often before FSHD myopathy is clinically evident, there may be no clinical symptoms to alert the physician of visual threat—especially in very young children who may not complain of significantly blurred vision. It is therefore frequent practice to advise periodic examinations by indirect ophthalmoscopy (a very brief and non-invasive investigation) by an experienced retinal ophthalmologist. This will obviously not of itself lead to early detection of exudates in premyopathic sporadic cases of FSHD, but there have certainly been successfully treated cases, especially in the USA, of retinal disease on screening young patients with familial FSHD (personal communications). Given that blindness in the affected eye is the typical consequence of untreated exudates impinging on the macula, I would argue that such surveillance is amply justified, notwithstanding the relative rarity of the clinical complication. Frequent surveillance of the clinically unaffected eye is also essential if one eye shows signs of exudative retinopathy, because bilateral retinal disease does occur.

13.2.2 Male sex bias in sporadic Coats' disease and in FSHD

FSHD appears to affect males earlier, more frequently, and probably on average more severely, than females (Zatz *et al.*, 1998). Furthermore, males who are FSHD mosaic typically have clinical muscle disease, whereas mosaic females are more likely to be unaffected 'carriers' (van der Maarel *et al.*, 2000). This could be due to a greater female sex-determined 'tolerance' for the adverse effects of the FSHD gene.

There is no significant evidence at present to suggest that clinical Coats' disease occurs more frequently or severely in FSHD-affected males than females, but outside this context there is a striking sex-bias in 'sporadic' Coats' disease, with 95% of such sporadic cases occurring in young boys. This cannot be explained on the basis of the occasional cases of Coats' disease known to be due to mutation of the 'norrin' gene on the X-chromosome (Black *et al.*, 1999). There may be some unidentified common pathogenetic factor in the causal pathways of the muscle disease and eye abnormality in FSHD which correlates with this sex bias.

13.2.3 FSHD muscle and retinal diseases are both highly asymmetric

It is possible that only one limb (Uncini *et al.*, 2002) or one eye (Gurwin *et al.*, 1985; Fitzsimons *et al.*, 1987) is affected. Some possible pathogenic implications of this asymmetry are discussed below.

13.3 Retinal disease and pathogenesis

13.3.1 Norrie disease and Coats' disease can both be caused by mutation of a TGF-beta protein ('norrin')

Classic Norrie disease is an X-linked disorder presenting soon after birth with blindness, dysplastic retinae, pseudogliomatous ocular masses and developmental retinal vascular abnormality. Some patients later develop deafness, psychosis or mental retardation. It is

caused by mutation of a gene which codes for a protein ('norrin') with the cysteine-containing characteristics of extracellular transforming growth factors beta (TGF beta) (Chen *et al.,* 1993a; Meitinger *et al.,* 1993).

Norrie disease was considered quite distinct from Coats' disease until Black *et al.* (1999) reported a female patient with Coats' disease who gave birth to a son with bilateral Norrie disease, caused in both cases by norrin mutation. The genetic abnormality was present as a 'somatic mutation' in the mother's blind eye. A norrin mutation was then found in another eye after nine eyes affected by sporadic Coats' disease were screened for norrin mutation. This clearly demonstrated that the 'norrie gene' could cause 'Coats' disease' and so raised the question of whether other members of the large TGF-beta family are implicated in other cases of Coats' disease—which might include FSHD.

It is therefore of some interest that in an addendum to his original report on a family with Coats' disease and muscular dystrophy Small (1968) quoted a communication from Dr. F.C.Blodi noting that 'except for the muscular dystrophy, many of the features of this (retinal) disease are similar to Norrie's disease'.

The auditory pathway pathology, assumed on the basis of electrophysiology to be cochlear, in FSHD is not known. However, it is known that the 'knock-out' mouse model of Norrie disease suffers from progressive hearing loss (as do many Norrie disease patients) and that this deafness is associated with progressive loss of blood vessels in the stria vascularis of the cochlea (Rehm *et al.,* 2002). It is difficult not to draw analogy with the developmental retinal vascular abnormalities, with 'drop-out of blood vessels', in retinae affected by Coats' disease and by Norrie disease. The Norrie 'knock-out mouse' also exhibits fundamental developmental malformations of the retinal vasculature (Richter *et al.,* 1998).

Some Norrie disease patients suffer from psychosis or mental retardation, which are not general associations of FSHD. It is debatable whether true mental retardation occurs in association with FSHD. Pseudo-mental-retardation is almost certainly much more commonly associated in infancy and early childhood than true mental retardation (see above). FSHD patients are in fact particularly well represented in occupations requiring mental agility. It is therefore mandatory that paediatricians be alert to the possibility of FSHD presenting as pseudo-retardation.

Identification of the gene mutated in Norrie disease has widened the spectrum of known retinal disorders which may sometimes be 'lumped' with Norrie disease rather than 'split' from it. Thus, X-linked familial vitreoretinopathy (FEVR), previously considered clinically distinct, may be caused by norrin gene mutation (Chen *et al.,* 1993a; Shastry *et al.,* 1997a). The question therefore arises as to whether clin-ically similar but autosomal dominant FEVR (Ober *et al.,* 1980) is caused by a different member of the TGF-beta protein family or a related factor. The severity of retinopathy of prematurity (ROP) may also be affected by norrin gene mutation (Shastry *et al.,* 1997b).

The fact that such different retinal vascular disorders are all caused or accentuated by mutation of 'norrin' implies that this TGF-beta protein has a fundamental role in determining various aspects of normal retinal vascular patterning during development. Indeed, such TGF-beta proteins and their associated signalling proteins are known to be important in developing vascular characteristics (Miyazono, 2002) and also myogenesis (see above).

Quite clearly, mutation of the norrin gene on the X chromosome cannot be primarily responsible for the telangiectasis and Coats' disease associated with FSHD, which is due to chromosome 4q35 mutation. However, since such telangiectasis and 'Coats' disease' is clinically indistinguishable from that which occurs 'sporadically', and since the 'TGF-beta super-family' incorporates some 30 different proteins (Miyazawa *et al.*, 2002), which in turn interact with a highly complex suite of signalling proteins, including 'Smads' (De Angelis *et al.*, 1998; Massague, 1998; Wooton *et al.*, 1999a, 1999b; Oh *et al.*, 2000; Pena *et al.*, 2000; Liu *et al.*, 2001; Hayashida *et al.*, 2003; Li *et al.*, 2004; Tajima *et al.*, 2003) , there is a possibility that another of these proteins is mutated or abnormal in FSHD with telangiectasis.

TGF-beta proteins and their receptors are also implicated in left-right patterning during development (Hamada *et al.*, 2002; Sakuma *et al.*, 2002), inflammation (Kunzmann *et al.*, 2003, Marino et al., 2003), endothelial activation (Goumans *et al.*, 2002) and muscle programming during development (Stern *et al.*, 1997; Bouche *et al.*, 2000). FSHD is characterized by sharply asymmetrical involvement of skeletal muscle, as well as of retinal vascular abnormality. Inflammation (Wulff *et al.*, 1982; Fitzsimons, 1994) occurs disproportionately to skeletal muscle destruction. Endothelial abnormalities (which may be related to inflammation) occur in FSHD muscle (Fitzsimons, 1999) and are thought to be the fundamental site of abnormality in Coats' disease (Tripathi and Ashton, 1971). Inflammation, which could well be primary (see discussion in Fitzsimons, 1994, 1999), occurs in Coats' disease (Tripathi and Ashton, 1971; Chang *et al.*, 1984).

Winokur *et al.* (2003) have demonstrated impaired oxidative responses in FSHD myocytes, and have suggested that FSHD might result from a fundamental flaw in myocyte maturation. So might selective muscle involvement in FSHD reflect a selective impairment in muscle regenerative capacity in some muscles rather than selective muscle fibre destruction? If so, what might determine variation in satellite cell/myoblast potential between muscles?

TGF-beta proteins regulate transcription via interaction with factors which repress such transcription, including DNA-binding proteins (Lam *et al.*, 2003; Wooton *et al.*, 1999a, 1999b). It has been proposed that inappropriate gene activation in FSHD is caused by derepression due to alteration in a DNA-binding complex caused by abnormal DNA (Gabellini *et al.*, 2002). However, aberrations of the transcription process might be caused by other mechanisms, perhaps involving growth factors.

Furthermore, if satellite cells and the myoblasts from which they derive are indeed abnormal, this could in turn reflect the interactions of the initial cell lineage during development with its surrounding extracellular matrix (Ramirez and Rifkin, 2003). Growth factors, including the members of the TGF-beta family and fibroblast growth factor (FGF) are known to affect myoblast characteristics, including proliferation and migration during development (Zantella and Massague, 1992; Florini and Magri, 1989; Pena *et al.*, 2000; Liu *et al.*, 2001; Riquelme *et al.*, 2001). Decorin, an extracellular matrix protein which is underexpressed in FSHD (Winokur *et al.*, 2003) also affects myoblast migration during development (Olguin *et al.*, 2003). Overexpression of FGF has been reported in one severe case of FSHD (Saito *et al.*, 2000).

The gross hypertrophy of some muscle fibres, which is a characteristic of FSHD muscle biopsies not shared with other dystrophies (Dubowitz, 1985), might also imply aberration of growth factors.

Whether or not there are any fundamental abnormalities of growth factors in FSHD remains to be seen. But the fact that, 13 years after the FSHD mutation was identified, the fundamental biochemical abnormalities consequent upon that mutation remain an enigma, clearly suggests that new approaches are needed and that clues should be sought in other ways. Perhaps the curious clinical features of FSHD and its associated retinal disease can provide us with those clues.

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14.
Unusual clinical features associated with FSHD

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

14.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is a dominantly inherited myopathy usually associated with a deletion of 3.3 kb *Kpn*I repeated units (D4Z4) on chromosome 4q35 (FSHMD1A; MIM 158900).

Typical clinical symptoms are characterized by unique involvement of muscles, which usually progress in a descending manner, including weakness and atrophy of facial muscles, followed by the shoulder girdle, the scapula fixators, and the upper arm muscles. Subsequently, pelvic girdle and lower limbs are also involved, and eventually, some 20% of the patients become wheelchair-bound by the age of 40 years (Lunt and Harper, 1991). Difficulties in whistling, closing the eyes, or lifting arms overhead are common initial symptoms. Prominent scapular winging and horizontally positioned clavicles are also observed. Facial or shoulder girdle weakness usually appears in adolescence, but signs may be apparent on examination in early childhood. Asymmetry of muscle involvement is often observed in apparently affected patients, but not related to handedness (Tawil *et al.,* 1994). Weakness is relatively mild and the progression is usually quite slow.

The clinical diagnosis of FSHD is sometimes difficult because the onset of the disease and the phenotypic expression is extremely variable, both within and between families (Lunt *et al.,* 1995; Padberg *et al.,* 1995b). One family may show severe disabilities with involvement of organs other than skeletal muscles, whereas others remain almost asymptomatic. Recent reports have shown much broader clinical expression of FSHD than perhaps previously recognized. In this chapter, unusual clinical features of FSHD are reviewed.

14.2 Early-onset form of FSHD

FSHD is generally a benign, slowly progressive myopathy that begins in late childhood or adolescence, and leads to disability only late in its course. However, some patients exhibit clinical symptoms from infancy or early childhood.

Korf *et al.* (1985) reported six patients in whom facial diplegia occurred in the first year of life, with subsequent development of facioscapulohumeral dystrophy. All had severe progressive disability prior to adolescence. Facial involvement did not include extraocular muscles. All six patients had a sensorineural hearing loss (Korf *et al.*, 1985). Bailey *et al.* (1986) also reported clinical, electrodiagnostic, and biopsy findings in a family with infantile FSHD. Four of eight family members having the disorder, all with onset in infancy, developed severe weakness leading to death in adolescence (Bailey *et al.*, 1986).

Although these reports have suggested that progressive and severe infantile FSHD is a genetically different form of FSHD, Brouwer *et al.* (1994) proposed them to be part of a wide clinical spectrum of FSHD. They designated the criteria for the early-onset form of FSHD as follows: (1) signs or symptoms of facial weakness before the age of 5 years; and (2) signs or symptoms of shoulder girdle weakness before the age of 10 years (Brouwer *et al.*, 1994).

Clinical features of the early-onset patients are similar but more severe, progressive, and variable (Kilmer *et al.*, 1995; Funakoshi *et al.*, 1998; Yamanaka *et al.*, 2002). Patients present with early-onset facial weakness or diplegia (Shapiro *et al.*, 1991; Jardine *et al.*, 1994a). Gait disturbances are observed before 28 years of age, and significantly earlier than the other group (Shapiro *et al.*, 1991; Jardine *et al.*, 1994a; Yamanaka *et al.*, 2002). Furthermore, the early-onset patients are often accompanied by bilateral sensorineural hearing loss, retinal vasculopathy, mental retardation and epilepsy (Shapiro *et al.*, 1991; Brouwer *et al.*, 1994; Funakoshi *et al.*, 1998; Miura *et al.*, 1998; Yamanaka *et al.*, 2002).

Nakagawa *et al.* (1997) detected the early-onset form in 17% of Japanese FSHMD1A patients. Yamanaka *et al.* (2002) estimated the frequency of early onset type of FSHD to be 13.4% (31/231 Japanese FSHMD1A patients from 145 unrelated families), and that was seen more frequently in sporadic cases. Genetic analysis revealed that they had significantly larger gene deletions on chromosome 4q35, and the patients with the smallest size of *Eco*RI fragment (10–11 kb) were usually of the early-onset type (Kilmer *et al.*, 1995; Funakoshi *et al.*, 1998; Yamanaka *et al.*, 2002).

14.3 Unusual muscle involvement observed in FSHD patients

14.3.1 Facial-sparing scapular myopathy

Scapular winging due to involvement of scapula fixators is a hallmark feature of FSHD, but may also be a prominent finding in other muscular disorders including Emery-Dreyfuss muscular dystrophy, congenital myopathies, myotonic dystrophy and acid maltase deficiency (Barohn *et al.*, 1993; Kissel, 1999). In the absence of facial muscle involvement, a diagnosis of FSHD would be difficult.

Jardine *et al.* (1994b) described a 4q-linked family including seven affected individuals in two generations. The patients showed scapular onset muscular dystrophy without facial involvement. Weakness began in the shoulders between 12 and 40 years of age. There was no distal weakness in the upper or lower extremities and there were no sensory abnormalities. In several cases, there was marked asymmetry with weakness on the right side more than on the left. There was no demonstrable facial weakness in any of the affected individuals.

Felice *et al.* (2000) performed genetic analysis on 14 patients with facial-sparing scapular myopathy, and determined that 71% of them had a short *Eco*RI fragment of less than 40 kb. These patients were estimated to constitute approximately 15% of FHSD patients. The clinical symptoms of the patients other than facial muscle involvement resembled typical FSHD patients in age at onset, physical characteristics, and association between fragment size and disease severity.

14.3.2 Tongue atrophy

Although involvement of facial muscles occurs in the majority of patients with FSHD, weakness of extraocular, masticatory, pharyngeal and lingual muscles are considered to be the exclusion criteria of the disease. However, some reports described the involvement of the tongue. Shimizu *et al.* (1991) reported a patient with 'congenital FSHD' with tongue atrophy. His father showed similar but milder muscle atrophy of the face and shoulder girdle since adolescence. The patient presented facial muscle weakness since birth, and then developed wasting around the neck, shoulder girdle, upper arms and thighs. Calf hypertrophy was also observed. Hearing disturbance was detected at the age of 6 years, and he also noted atrophy of the tongue and the bilateral thighs at the age of 10. EMG in the extremities and the tongue revealed myopathic changes. Goto and Sugihara (1994) also reported a patient diagnosed as congenital FSHD. Her mother had moderate facial weakness and mild proximal weakness of the upper and lower limbs. The patient presented bilateral facial weakness, tongue atrophy and weakness of the shoulder girdle, upper arms, and thighs, and bilateral mild sensorineural hearing loss.

Yamanaka *et al.* (2001) observed that 4.6% (7/151) of Japanese patients with FSHMD1A had tongue atrophy with abnormal MRI findings and typical myogenic patterns of electromyography *(Figure 14.1)*. All seven patients belong to a group of early-onset FSHD and the *Eco*RI fragment size varied from 10 to 17 kb. They suggested that the FSHD patients, especially with a large gene deletion on chromosome 4q35 could have myopathic tongue atrophy.

14.3.3 Head drooping

Ichikawa *et al.* (1996) described three unrelated patients with FSHD showing conspicuous head drooping caused by severe wasting of posterior neck muscles. These patients realized abnormal neck posture much earlier than appearance of obvious gait disability, while they show other characteristic FSHD features. Other affected members from the same families did not show abnormal head drooping.

14.3.4 Abdominal muscle involvement and lumbar lordosis

Awerbuch *et al.* (1990) reported that the Beevor sign is commonly observed in patients with FSHD but not in other types of neuromuscular disorder. This sign was originally proposed by English neurologist C.E.Beevor as an indication of the level of involvement in spinal cord lesions. The umbilicus moves upward when the subject in the supine position raises their head because of weakness of the lower

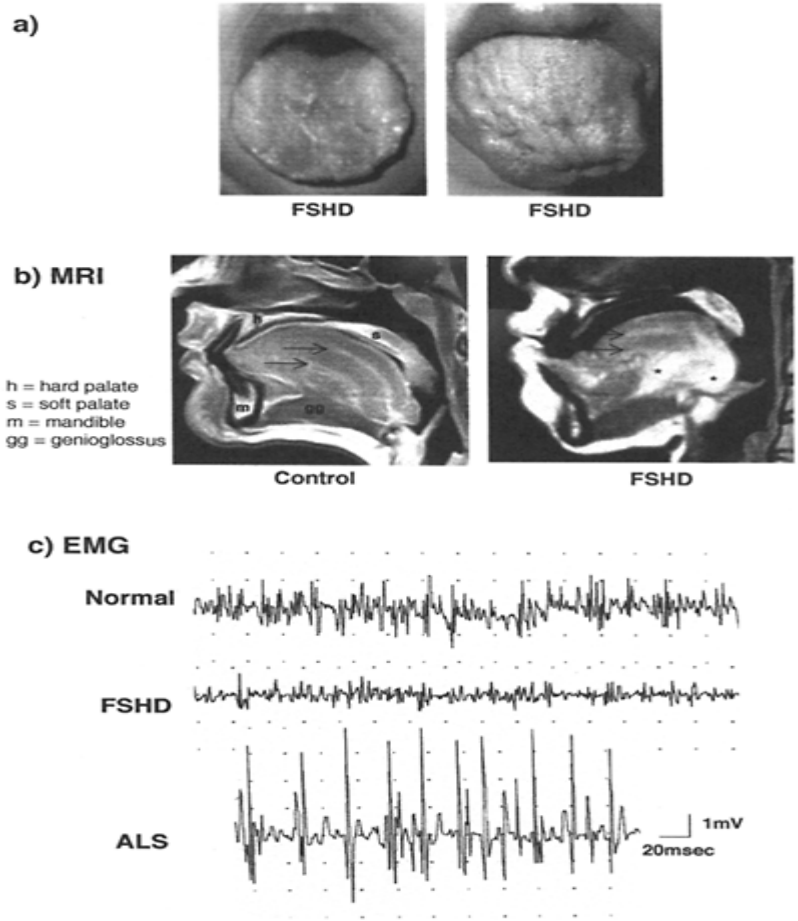

Figure 14.1 (a) Tongue pictures of FSHD patients with tongue atrophy (Yamanaka *et al.*, 2001). (b) Tongue MRI of a normal control and an FSHD patient. The normal tongue virtually

fills the entire oral cavity. Its internal structure shows two curvilinear bands (arrows) parallel to the mucosal surface. In FHSD, there are scattered abnormal high-intensity areas (asterisks) in the internal tongue structure. The two curvilinear bands (arrows) are deranged and show disorganization of the tongue architecture. The atrophic tongue produces a space in the upper oral cavity (Yamanaka *et al.,* 2001). (c) In the FSHD patient, EMG showed typical myogenic changes, whereas a patient with amyotrophic lateral sclerosis (ALS) showed neurogenic changes.

rectus abdominis muscles. In FSHD patients, this sign appears even before functional weakness of abdominal wall muscles is apparent.

Early involvement of the abdominal muscles with relative sparing of the psoas major muscle in FSHD patients was detected by CT scan (Horikawa *et al.,* 1992). This may exacerbate lumbar lordosis, the most common form of spinal deformity in the patients (Kilmer *et al.,* 1995).

14.3.5 Limb girdle type muscle weakness

Limb girdle muscular dystrophy (LGMD) is a group of genetically heterogeneous progressive muscular disorders predominantly involved in proximal limb muscles. The genes responsible and their protein products have been identified in at least three autosomal dominant and ten autosomal recessive forms.

Some 4q35-linked FSHD patients were reported to display limb girdle type muscular weakness. The initial symptoms of the patients were weakness of the proximal lower limb muscles, and complaint of difftculty in climbing stairs and walking. Facial muscle involvement was absent or very mild (Nakagawa *et al.,* 1996, 1997; van der Kooi *et al.,* 2000; Felice and Moore, 2001). Although detailed genetic analysis was not performed, Kazakov and Rudenko (1995) also reported a clinically and genetically homogeneous group of patients with autosomal-dominant inheritance manifesting a gradually descending form of FSHD, called facioscapulolimb dystrophy (FSLD).

Reardon *et al.* (1991) reported a 32-year-old male patient who had typical calf hypertrophy and limb girdle type of muscle weakness. These findings suggested Becker muscular dystrophy (BMD), although he presented with sudden onset of facial weakness at age of six. When his daughter showed facial weakness, autosomal dominant FSHD

became most likely. Since calf hypertrophy, although rare, has been reported in FSHD, differentiation between FSHD and BMD may also be difficult in an isolated male patient.

14.3.6 Distal myopathy

Some patients with FSHD presented foot drop by virtue of weakness of the foot extensor muscle. Padberg investigated 107 patients and found foot extensor weakness in 8% (Padberg, 1982). Felice and Moore (2001) reported a 78-year-old woman who was followed for 15 years with a diagnosis of late-onset autosomal dominant distal myopathy. The patient showed progressive bilateral foot drop, and later developed difficulties climbing stairs. Although mild eye-closure weakness and late-onset sensorineural hearing loss were observed, the patient showed no other clinical characteristic features of FSHD. Her mother had similar problems with mild facial muscle involvement and hearing loss. The patient was believed to have a form of hereditary distal myopathy, although genetic analysis revealed this proband to have a 30 kb *Eco*RI fragment.

Involvement of calf muscles is presumed to be affected only in later stages of the disease as compared with the anterior tibial muscle. CT scans also revealed a relatively mild involvement of the gastrocnemius and soleus muscles as compared with the tibialis anterior muscle (Horikawa *et al.*, 1992). However, van der Kooi *et al.* (2000) reported a male patient who initially experienced foot pain and inability to walk on his toes at the age of 50 owing to calf muscle involvement. The *Eco*RI fragment size of this patient was 20 kb.

14.4 Muscle pain

Muscle pain is rarely described as a symptom of FSHD. Some reports described the association of muscle pain and weakness of facioscapulohumeral distribution, but they were unable to distinguish between FSHD and polymyositis (Rothstein *et al.*, 1971; Munsat *et al.*, 1972; Bates *et al.*, 1973; Bacq *et al.*, 1985). van der Kooi *et al.* (2000) reported a patient showing mild shoulder symptoms such as tiredness and pain. The *Eco*RI fragment size exhibited by this patient was 38 kb (van der Kooi *et al.*, 2000). These authors also described another patient with a 20 kb *Eco*RI fragment who initially exhibited foot pain and calf muscle weakness.

Bushby *et al.* (1998) reported four adult patients with FSHD showing muscle pain, which remains their most disabling symptom. Three of them had a short *Eco*RI fragment from 20 to 24 kb, but one manifested only normal-sized fragments. All patients reported between three and seven different pains of varying site and nature, and none had more than one painfree day per month and all complained of disturbed sleep. These myalgic pains could be particularly difficult to control by analgesic or anti-inflammatory therapy. They concluded that muscle pain in FSHD is an under-reported but significant symptom.

14.5 Association with other types of neuromuscular disorders

Tonini *et al.* (2002) reported two unique Brazilian families with FSHMD1A and other forms of muscular dystrophy in the same family. In the first, the 35-year-old male proband had limb girdle muscular dystrophy with proximal weakness, elevated creatine kinase and a myopathic muscle biopsy. All the proteins known to be associated with limb girdle muscular dystrophy were normal. Two of his sisters also complained of muscle weakness. The oldest sister exhibited clinical signs consistent with FSHD, and had a 30 kb *Eco*RI/*Bln*I fragment which was found in another six relatives, but surprisingly not in the affected proband or the other sister. In the second family, a 57-year-old male with a typical FSHD phenotype had a 17 kb *Eco*RI/*Bln*I fragment which was also present in other affected relatives. However, in a 14-year-old severely affected male cousin, confined to a wheelchair since age 12, but without facial weakness, the small fragment was absent. In case of these rare associations, it may be important to perform genetic tests in all affected individuals in a family.

Sakuma *et al.* (2001) reported a male patient with FSHD accompanied by myasthenia gravis. The patient had a 35 year history of FSHD and his mother was also affected. At the age of 50, the proband was admitted to hospital because of acute progression of muscle weakness without any fluctuation. No blepharoptosis or ocular movement disturbance was observed. However, disturbance in chewing and swallowing appeared about a month after admission, an uncommon finding in FSHD. The diagnosis of myasthenia gravis was confirmed by the repetitive stimulation test, edrophonium chloride injection, and by titre of serum anti-Ach receptor antibody (Sakuma *et al.,* 2001).

14.6 Cardiac involvement

Involvement of cardiac muscle and serious electrocardiographic abnormalities are rare in FSHD, and are not considered to be part of the disease (de Visser *et al.,* 1992; Kilmer *et al.,* 1995). However, some reports have described FSHD patients with serious arrhythmia. These patients required pacemaker implantation because of symptomatic atrial tachycardia or complete A–V block (Ohno *et al.,* 1991; Shen and Madsen, 1991).

Also, a unique family with both FSHMD1A and hereditary long QT syndrome (LQT) was reported. In this family, five individuals in three generations were diagnosed as having FSHMD1A by clinical and genetic analysis, and three of the five affected members were also diagnosed as having LQT. LQT constitutes a group of disorders that cause syncope and sudden death from ventricular arrhythmia in an autosomal dominant fashion. One of the loci for LQT (LQT4) was mapped to chromosome 4q25-q27, and possible linkage between FSHD and LQT was speculated upon (Kimura *et al.,* 1997). Recently, an ankyrin B gene mutation was identified in the large French family with LQT4 (MIM: 600919) (Mohler *et al.,* 2003). Genetic analysis should clarify the association of FSHD and LQT4 in this family.

Possible cardiomyogenic involvement in FSHD was also reported using Thallium-201 single-photon-emission computed tomography (Tl-201-SPECT). Yamamoto *et al.* (1986)

reported abnormal reduced Tl-201 uptake in 71% of FSHD patients that were scattered in all left ventricular wall segments. Faustmann *et al.* (1996) revealed stress-induced reduced Tl-201 uptake in the affected members of a 4q35-linked FSHD family and concluded that careful supervision of cardiac functions may be needed for FSHD patients.

Further, the thoracic deformity observed in FSHD may cause cardiac problems. Nakayama *et al.* (1999) studied electrocardiogram (ECG) and ECG-gated cardiac magnetic resonance imaging (MRI) in eight patients with FSHD. The patients frequently showed ECG abnormalities including elevated P wave and multifocal atrial premature contractions, together with restricted right ventricular movement and enlarged right atrium. Similar changes are often observed in patients suffering from severe 'funnel chest' or 'straight back syndrome'. The authors concluded that the characteristic thoracic deformity may play a primary role in the development of cardiac problems in FSHD (Nakayama *et al.*, 1999).

14.7 Respiratory failure

Pulmonary dysfunction in FSHD is usually mild, if present. However, some patients show progressive, life-threatening respiratory failure. Yasukohchi *et al.* (1988) described two sibs with FSHD. The 8-year-old sister had only muscle manifestations, whilst the brother, aged 13 years, manifested sensorineural hearing loss and marked tortuosity of retinal arterioles. He also showed early onset and progression of severe restrictive pulmonary dysfunction, and cor pulmonale, which led to death. Nakagawa *et al.* (1996) also reported an early-onset FSHD patient with respiratory failure and retinal vasculopathy. Interestingly, the 53-year-old mother of the proband with the same genetic abnormality had limb girdle type muscular weakness with very mild facial involvement.

Kilmer *et al.* (1995) reported that nearly 50% of the FSHD patients had vital capacity evidence of restrictive lung disease. However, only 13% had severe involvement, and only 22% had a history of pulmonary complications. There was no age or disease duration effect on pulmonary function measurements or complications. They concluded that maximal expiratory pressure measurements on the FSHD patients were more sensitive than other pulmonary function tests, as with the other neuromuscular diseases (Kilmer *et al.*, 1995).

14.8 Central nervous system involvement

FSHD patients usually exhibit little or no cognitive impairment (Sigford and Lanham, 1998). However, the patients with a large deletion in the FSHD gene region tend to have a higher chance of showing severe clinical phenotypes with central nervous system abnormalities. Akiyama *et al.* (1991) reported a female patient with FSHD with sensorineural deafness, retinal vessel abnormality, mental retardation and epilepsy with infantile spasms at 6 months of age.

Miura *et al.* (1998) reported two unrelated, severely affected patients with mental retardation and epilepsy. One patient showed infantile spasms at the age of 4 months and

localization-related epilepsy at the age of 2.5 years. Muscular atrophy in the face, shoulder girdle and upper arms was observed from the age of 4 years. In the other patient, lack of facial expression was noticed from the age of 1 year, and at 4 years she was noted to have a loss of bilateral upward gaze. She developed localization-related epilepsy at the age of 9 years. From the age of 10 years, weakness of the lower limbs progressed and she became wheelchair-bound at the age of 14 years and 8 months. She had moderate sensorineural hearing loss, a loss of bilateral upward gaze and tongue atrophy. Their IQs were 33 and 45, respectively. Southern blot analysis revealed a 10 kb *Eco*RI fragment in both patients (Miura *et al.*, 1998).

Funakoshi *et al.* (1998) found nine patients with the smallest *Eco*RI fragments (10–11 kb), and reported that all of them were classified as having the early-onset form. These patients exhibited a high frequency of both epilepsy (4/9, 44%) and mental retardation (8/9, 89%). These two reports concluded that mental retardation and epilepsy may be part of the clinical spectrum of FSHD, especially in the early-onset form with a large deletion.

14.9 Psychopathological and emotional examination

Bungener *et al.* (1998) performed psychological studies on patients with 11 FSHD, together with 15 myotonic dystrophy and 14 healthy subjects. A semistructured interview was used to determine DSM III-R criteria for major depressive episodes, dysthymic episodes, and generalized anxiety. The Montgomery and Asberg, and the Hamilton depressive scales, the Covi and Tyrer anxiety scales, the Abrams and Taylor scale for emotional blunting, and the depressive mood scale were all used in the study. The results indicated that the patients with FSHD were the most depressed and most anxious.

14.10 Hearing loss

Meyerson *et al.* (1984) reported sensorineural hearing loss in two sibs with FSHD. Gieron *et al.* (1985) described a mother and three children with FSHD, sensorineural hearing loss, and marked tortuosity of retinal vessels. The deafness, which varied from mild to moderate, was bilateral and early in onset; audiological studies indicated the cochlea to be the site of the abnormality. Matsuzaka *et al.* (1986) reported a sporadic patient with early-onset FHSD, sensorineural hearing loss, mental retardation, and marked tortuosity of the retinal arterioles. Fujimura *et al.* (1989) also reported a sporadic case of a 12-year-old boy with FSHD, sensorineural hearing loss and exudative angioma of bilateral retina. His hearing loss was noted at 9 years, followed by muscle weakness of his right upper extremity at 11 years.

Voit *et al.* (1986) found bilateral sloping high frequency hearing loss of 20–90 dB in 6/10 patients with infantile- or adolescent-onset FSHD. In some patients, the hearing loss was clearly progressive. The outer hair cells of the basal turn were predominantly affected. The authors concluded that cochlear dysfunction is a specific and frequent phenomenon of early-onset FSHD.

Brouwer *et al.* (1991) performed screening audiometry in 56 patients with autosomal dominant FSHD and suggested that the change of hearing function between 4000 Hz and

6000 Hz is part of the disease and may lead to severe hearing loss in some patients. Generally, the patients show bilateral high-tone hearing loss, but some showed also at the lower (speech) frequencies.

Brain stem auditory-evoked potentials were generally normal (Verhagen *et al.*, 1995), but some patients exhibited abnormal increased threshold and prolonged latency (Takeya *et al.*, 1990; Fierro *et al.*, 1997).

The frequency of hearing loss was estimated to be about 25–64% of affected patients, and is now considered to be an important feature of FSHD (Sanchez-Alcon *et al.*, 1994; Padberg *et al.*, 1995a). Age and severity of the myopathy did not have a clear relationship with the hearing loss (Sanchez-Alcon *et al.*, 1994; Padberg *et al.*, 1995a). On the other hand, Rogers *et al.* (2002) undertook detailed pure tone audiometric examination in 21 adult-onset FSHD cases and found no significant difference in the prevalence of hearing impairment. They concluded that hearing impairment is not common in adult-onset facioscapulohumeral muscular dystrophy. Moderate to severe sensorineural deafness is, however, common in early-onset FSHD.

14.11 Retinopathy

Retinal vasculopathy is known to be associated with FSHD. Association of FSHD and Coats' syndrome (exudative retinopathy with telangiectasis, sometimes causing blindness) was reported especially in the severe early-onset form with mental retardation (Small, 1968; Taylor *et al.*, 1982; Voit *et al.*, 1986).

Gurwin *et al.* (1985) reported a 22-year-old FSHD patient with a macular lesion in her right eye and poor central vision, which had been present since early childhood. Fluorescein angiographic examination revealed bilateral peripheral vessel closure, peripheral retinal telangiectasis, and hyperfluorescence in both foveae. Three affected family members also had clinical deafness and abnormal retinal vasculature, as determined by fluorescein angiography, but none had related visual symptoms. The authors concluded that in young patients with unexplained retinal vascular lesions, the diagnosis of FSHD should be considered (Gurwin *et al.*, 1985).

Fitzsimons *et al.* (1987) found peripheral retinal capillary abnormalities including telangiectasia, closure, leakage and microaneurysm formation in 56 of 75 individuals with clinical or genetic evidence of FSHD. Retinal vasculopathy may present early in life and before there is overt evidence of muscle disease. However, there was no correlation between the severity of the muscle disease and the extent of the retinal vascular abnormality (Fitzsimons *et al.*, 1987). Padberg *et al.* (1995b) also reported similar retinal vasculopathy including telangiectasia and microaneurysms in 49% of patients with FSHD by using fluorescein retinal angiography.

The risk to vision has not been established since there are only few reports of severe visual loss in FSHD. Pauleikhoff *et al.* (1992) reported two cases of young girls who developed FSHD and exudative retinal detachment due to telangiectasis. In the first patient, the severity of the disease precluded visual recovery despite extensive photo- and cryotherapy. In the other, visual acuity in both affected eyes was retained after treatment (Pauleikhoff *et al.*, 1992).

Since visual loss may be preventable, ophthalmic examination should be undertaken on infants and young children at risk of having a deletion of the FSHD region, although visual complications of telangiectasis are rare (Fitzsimons *et al.*, 1987; Pauleikhoff *et al.*, 1992).

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15.
Molecular diagnosis of FSHD

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

15.1 Introduction

Molecular diagnosis can confirm FSHD in most cases by the detection of D4Z4 repeat contraction on 4q35 (FSHD1) (Wijmenga *et al.*, 1992; recently reviewed by Upadhyaya and Cooper, 2002). Nowadays, diagnostic testing is increasingly used for the exclusion of patients with only mild neuromuscular presentation. However, the clinical variability and the genetic complexity of FSHD both complicate reliable diagnosis.

FSHD is characterized by a variable age of onset and a wide range of clinical severity both between and within families. In recent years, it has been demonstrated that hearing loss, epilepsy and mental retardation can be part of FSHD (Brouwer *et al.*, 1991, 1995; Funakoshi *et al.*, 1998; Miura *et al.*, 1998; Felice *et al.*, 2000; van der Kooi *et al.*, 2000; Felice and Moore, 2001). In addition, FSHD patients have been reported who do not manifest a contracted D4Z4 array on chromosome 4 and in whom the disease is not even linked to 4q35 (Gilbert *et al.*, 1992, 1993; Tim *et al.*, 2001).

Molecular testing for FSHD is complicated by interference from a highly homologous, but non-pathogenic repeat region on 10q26 (15.3). Furthermore 4; 10 D4Z4 translocations (15.4) and, as a result, the formation of hybrid D4Z4 repeat arrays (15.5) have hampered diagnostic testing. These complicated situations underscore the need for a reliable and if possible straightforward genetic test for FSHD, not merely as a confirmatory test but also as an exclusion test.

In this chapter, we discuss both the common and complicated FSHD genotypes that may be encountered during the molecular diagnosis of FSHD. For each situation, a diagnostic test is discussed based on standard linear electrophoresis. Most additional tests were initially developed using techniques allowing the separation of all D4Z4 fragments ((pulsed-field gel electrophoresis (PFGE) and field inversion gel electrophoresis (FIGE)). The different diagnostic tests introduced in each paragraph therefore focus on the use of FIGE and PFGE. Unfortunately, owing to the recombinogenic nature of the 4q35 and

10q26 repeats, it is not possible to resolve all diagnostic problems with a single test. Despite this, we hope that the methods offered will help to improve FSHD diagnostics.

15.2 General

FSHD is transmitted as an autosomal dominant trait. Linkage analysis assigned the major FSHD locus (FSHD1) to chromosome 4q35 and the disease is closely linked to D4F104S1 (Upadhyaya *et al.*, 1990; Wijmenga *et al.*, 1990, 1992). Within the FSHD1 locus, a highly polymorphic tandem repeat array comprising 3.3 kb D4Z4 units is located, that is highly susceptible to rearrangement (Wijmenga *et al.*, 1992). It is the contraction of this repeat array (i.e. the reduction in the number of D4Z4 repeat units) on one of the chromosomes 4 to below a critical threshold that is associated with the clinical expression of FSHD. The D4Z4 repeat array is visualized by Southern blotting of *Eco*RI-digested DNA followed by hybridization with probe p13E-11 (D4F104S1), which recognizes a region proximal to D4Z4. In the vast majority of FSHD patients, one *Eco*RI fragment from 4q35 is <38 kb (10 repeat units) whereas in healthy controls, the D4Z4 repeat array ranges from 41 to more than 335 kb (11–100 repeat units).

15.3 10q26 homology

DNA diagnosis is complicated by cross-hybridization of p13E-11 to a highly homologous polymorphic array on 10q26 (Bakker *et al.*, 1995; Deidda *et al.*, 1995). Thus, probe p13E-11 (D4F104S1) identifies a total of four polymorphic *Eco*RI restriction fragments, two of which are derived from 4q35 and two from the homologous locus on 10q26. Furthermore, probe p13E-11 cross-hybridizes to a 9.4 kb fragment of chromosome Y. At least 10% of chromosome 10-derived fragments in the population are <38 kb but have no pathogenic consequences (Bakker *et al.*, 1995).

Despite interference from the 10q26 D4Z4 arrays, molecular diagnosis in those sporadic FSHD cases in which a *de novo* D4Z4 rearrangement has been identified, were considered to be both accurate and reliable (Bakker *et al.*, 1996). For familial cases without a *de novo* fragment, however, the small 10q26-derived fragments served to complicate FSHD diagnosis.

The subsequent identification of a unique *Bln*I restriction site present only in 10q26-derived D4Z4 repeats allowed differentiation between 4q35- and 10q26-derived repeat arrays (Deidda *et al.*, 1996) *(Figure 15.1)*. After double digestion with *Eco*RI/*Bln*I, only 4q35 fragments remain visible, whereas those of 10q26 are cleaved by *Bln*I. Those *Eco*RI fragments, with a corresponding *Eco*RI/*Bln*I fragment of <38 kb, are considered to be chromosome 4-derived and are therefore indicative of FSHD *(Figure 15.2)*. The introduction of the differential *Eco*RI/*Bln*I double digest considerably improved the molecular diagnosis for the majority of FSHD (Bakker *et al.*, 1996; Deidda *et al.*, 1996; Upadhyaya *et al.*, 1997; Galluzzi *et al.*, 1999).

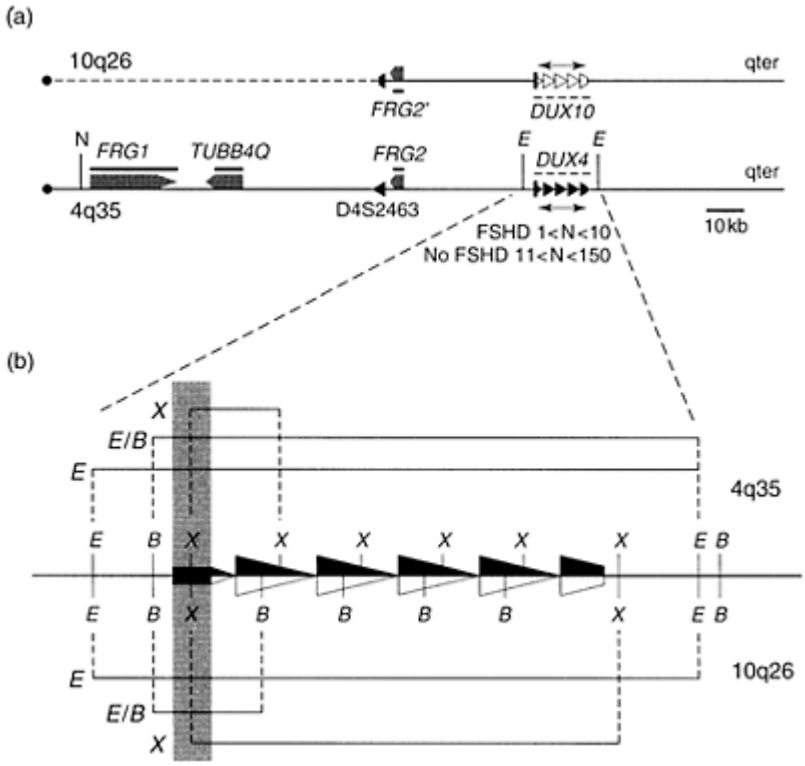

Figure 15.1a Schematic diagram of chromosome 4q35 encompassing the FSHD candidate region as well as the homologous region of chromosome 10q26. FSHD is a dominantly inherited disorder caused by contraction of the D4Z4 repeat array (triangles) on one of the chromosomes 4. D4Z4 contractions on chromosome 10 are not associated with FSHD. Normally, the polymorphic D4Z4 repeat varies between 11 and 150 units, while FSHD patients carry one allele with 1–10 units. It is generally hypothesized that D4Z4 contraction alters the chromatin structure resulting in transcriptional deregulation, most likely in distal 4q.

Four (pseudo-) genes identified in or close to the D4Z4 repeat on 4q35 are depicted (*FRG1, TUBB4Q, FRG2* and *DUX4*). The locations of the *Eco*RI (E) restriction sites adjacent to D4Z4 and probe p13E-11 (solid box) are shown. Chromosomal assignment described in Section 15.9 makes use of the *Not*I restriction site (N) proximal to *FRG1*.

Figure 15.1b Schematic presentation of the D4Z4 locus on chromosome 4 (solid) and its homologue on chromosome 10 (open). The repeat units are represented by a triangle. The region recognized by probe p13E-11 is shaded. The restriction sites for *Eco*RI (E), *Bln*I (B) and *Xap*I (X) are indicated. Above the D4Z4 repeat array, solid bars visualize the restriction fragments, after digestion with the proper enzymes for 4qter. Below the D4Z4 repeat array, the restriction fragments for 10qter are visualized.

15.4 4;10 D4Z4 translocations

Owing to the large fragment sizes, visualization of all polymorphic D4Z4 fragments of 4q35 and 10q26 can only be achieved by FIGE or PFGE, allowing separation of fragments of >50 kb. The application of PFGE on control DNA samples from the Dutch population revealed the existence of 4; 10 D4Z4 translocations: 4-derived arrays on chromosome 10 or *vice versa* (10-derived arrays on chromosome 4), in some 21% of individuals *(Figure 15.3)* (van Deutekom *et al.,* 1996; Lemmers *et al.,*

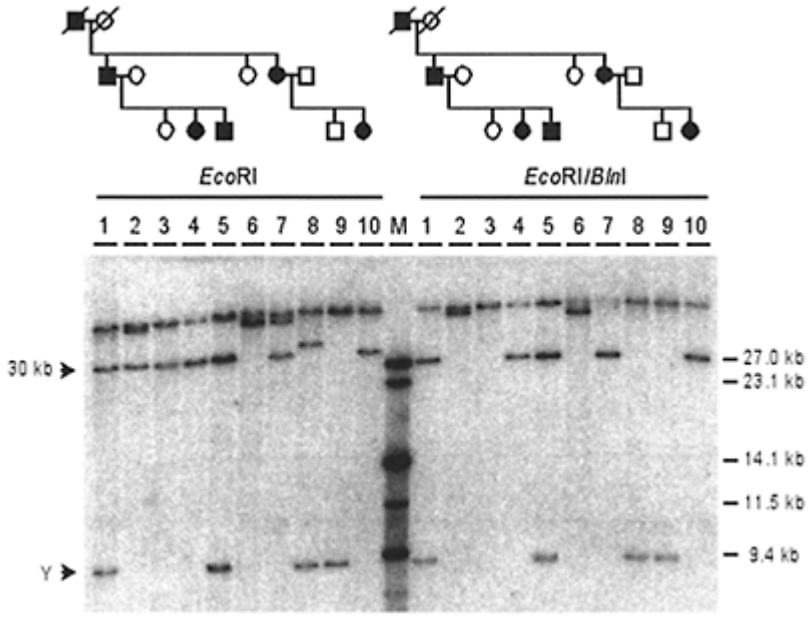

Figure 15.2 Molecular analysis of FSHD in family 87 by linear gel electrophoresis of *Eco*RI and *Eco*RI/*Bln*I digested DNA followed by Southern blot analysis using probe p13E-11. An approximately 30 kb *Eco*RI fragment is observed in affected and unaffected family members (left). After *Eco*RI/*Bln*I double digestion (right), this 30 kb fragment is 3 kb reduced in the affected members only, indicating 4q35 origin. The co-migrating 30 kb 10q26 fragments in unaffected individuals are completely fragmented by this digestion, thereby unequivocally identifying the FSHD alleles in the patients.

1998; van Overveld *et al.,* 2000). From recent publications, it becomes clear that 4; 10 D4Z4 translocations can be encountered worldwide and that the ratio of 4; 10 vs. 10; 4 translocations can vary between different ethnic groups (Matsumura *et al.,* 2002).

4; 10 translocations can complicate DNA diagnosis, especially in the case of an apparent chromosome 10-like allele <38 kb detected by conventional gel electrophoresis. It was reported that these FSHD-sized 10-type alleles can be pathogenic when translocated to chromosome 4 (Deidda *et al.,* 1996; van Deutekom *et al.,* 1996). However, new genetic tests have shown that these pathogenic 10-type repeat arrays are mixtures of 4- and 10-type repeat units, i.e. hybrid alleles (see Section 15.5). FSHD-sized translocated homogeneous chromosome 10 repeats on chromosome 4 have so far never been encountered in FSHD (Lemmers *et al.,* 2001; Zhang *et al.,* 2001).

Most 4; 10 translocations can not be detected with conventional gel electrophoresis since this technique does not allow separation of large D4Z4 fragments. This shortcoming necessitates the use of FIGE or PFGE, which is unfortunately not available in most diagnostic laboratories. In order to overcome this problem, a modified Southern blot-based *Bgl*II/*Bln*I dosage test has been developed (van der Maarel *et al.,* 1999) that does not require PFGE (see Section 15.10).

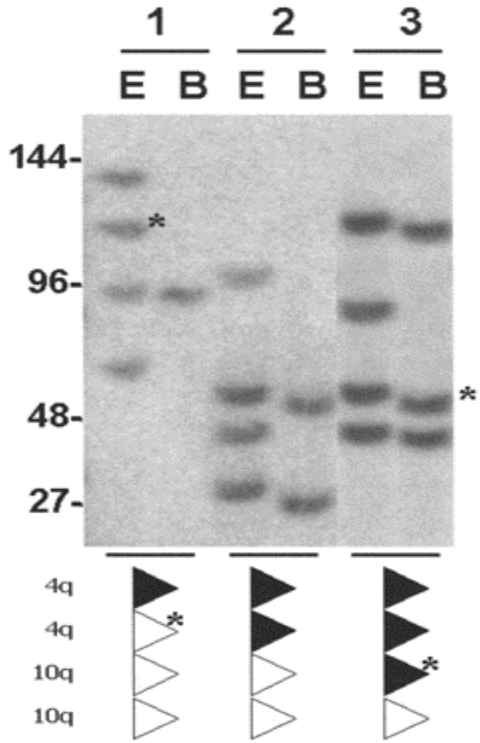

Figure 15.3 PFGE analysis of the D4Z4 repeat array revealed the presence of 4; 10 translocations as shown for individuals 1, 2 and 3. DNA of these three cases was double

digested with *Eco*RI/*Hind*III (E) and *Eco*RI/*Bln*I (B) and hybridized with p13E-11. In PFGE analyses, *Hind*III is added to the *Eco*RI restriction since this enzyme increases the resolution in the 20–50 kb range and does not cut within the repeat array itself. D4Z4 allele constitutions are depicted below the blot and translocated alleles of individuals 1 and 3 are marked with an asterisk. Individual 1 has three 10-type alleles. Apparently, one of the chromosome 4 alleles carries a 10-type D4Z4 repeat array. The DNA of individual 2 shows no translocated alleles. Individual 3 has one chromosome 10 with a translocated 4-type D4Z4 repeat array giving rise to an allele constitution of one 10-type and three 4-type D4Z4 alleles. D4Z4 allele constitutions as represented by individuals 1 and 3 are both observed in about 10% of the Dutch population. The D4Z4 allele constitution of individual 2 is most common (80%).

Although FIGE/PFGE or the dosage test can demonstrate the existence of 4; 10 translocations, the chromosomal origin of the translocated repeat arrays cannot unequivocally be determined with these methods. For this purpose, additional chromosome-specific probes hybridized to Southern blots of *Not*I-digested DNA are required. Determination of the chromosomal origin is described in Section 15.9.

15.5 Hybrid D4Z4 repeat arrays

The previous paragraph described the identification of 4q; 10q translocations. In general, rearrangements of tandem repeat arrays like D4Z4, occur between sister chromatids or homologous chromosomes. Therefore, rearrangements between homologous chromosomes that carry D4Z4 repeat arrays from different chromosomal origins can cause the formation of hybrid alleles, consisting of mixtures of chromosome 4- and 10-derived units. Indeed, PFGE analysis revealed the presence of hybrid D4Z4 repeat arrays

(Cacurri *et al.,* 1998; Lemmers *et al.,* 1998). These hybrid alleles add to the complexity of molecular diagnosis for FSHD.

Hybrid alleles of <38 kb on chromosome 4 are pathogenic, like the homogeneous 4-type D4Z4 repeat arrays. No FSHD patients have been reported with short hybrid repeats on chromosome 10. Small hybrid alleles can be recognized by a reduction upon *Eco*RI/*Bln*I digestion that is larger than the usual 3 kb. However, large hybrid alleles can also give rise to small *Eco*RI/*Bln*I fragments when they possess distal of D4F104S1, a small cluster of chromosome 4 units followed by chromosome 10 units. When these alleles accidentally co-migrate with small 10-type alleles, they can appear like FSHD-sized allele and can therefore lead to false-positive FSHD diagnosis as demonstrated in *Figure 15.4a* (lane *Eco*RI and *Eco*RI/*Bln*I).

Even using PFGE or FIGE it is not possible to allow determination of the original *Eco*RI fragment from which the short hybrid *Eco*RI/*Bln*I fragments are derived when only *Eco*RI and *Bln*I are used *(Figure 15.4b).* To overcome this problem, the restriction enzyme *Xap*I was introduced into FSHD diagnosis (Lemmers *et al.,* 2001). *Xap*I has opposite characteristics to *Bln*I: it recognizes a single restriction site within the 4-derived repeat unit, while the 10-derived repeat unit is *XapI*-resistant *(Figure 15.1).* Therefore, digestion of DNA with *Eco*RI, with *Eco*RI/*Bln*I and with *Xap*I, allows unequivocal determination of chromosome 4- and 10-derived alleles *(Figure 15.4).* Furthermore, this triple DNA analysis is useful to identify cases of identically sized 4-derived and 10-derived arrays (co-migrating alleles) *(Figure 15.5).*

Without any further adaptation, *Xap*I can be introduced into linear gel electrophoresis. Even without the characterization of all four repeat arrays, it will allow resolution of complicated D4Z4 genotypes. The increasing use of the DNA test as an exclusion criterion necessitates the additional use of *Xap*I to obtain complete allele information.

Hybrid repeat arrays are often found on 4; 10 translocated alleles. Assignment of the chromosomal origin of translocated alleles is described in Section 15.9.

15.6 D4F104S1 deletion

In a few FSHD patients, both sporadic and familial, the characteristic short 4q35-derived *Eco*RI fragment could not be visualized by routine diagnosis (Upadhyaya *et al.,* 1997). One explanation for these cases is the presence of other mutations unlinked to the FSHD locus at 4q35. In some cases, however, the apparent absence of a FSHD-sized *Eco*RI fragment may be due to the absence of the genomic region proximal to the D4Z4 repeat array that encompasses the p13E-11 (D4F104S1) probe (Lemmers *et al.,* 1998). The frequency in FSHD patients of these proximally extended D4Z4 deletions is currently unknown. Such extended deletions can lead to misinterpretation in the diagnostic setting, since they remain undetected with conventional gel electrophoresis and do not appear to be associated with a specific

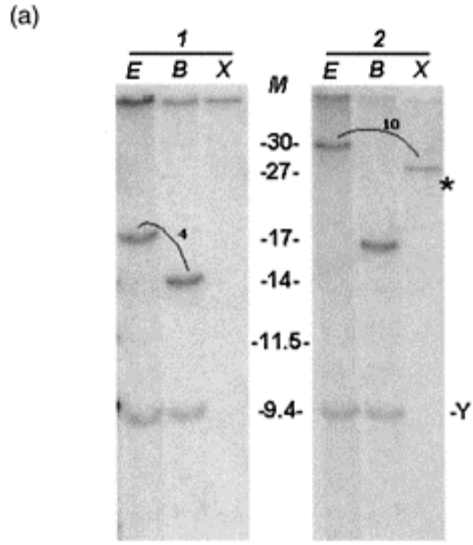

Figure 15.4a Triple DNA analysis of two possible FSHD patients. Fragment sizes in the marker lane (M) are indicated in the middle. The cross-hybridizing 9.4 kb Y chromosomal fragment is indicated on the right. DNA of these individuals was digested with *Eco*RI (E), *Eco*RI/*Bln*I (B) and with *Xap*I (X) and separated in adjacent lanes. The new informative *Xap*I fragment is indicated with an asterisk. Patient 1 carries a standard FSHD allele of 17 kb. The fragment is resistant to *Bln*I digestion, gets reduced to 14 kb due to the *Bln*I site 3 kb distal to the proximal *Eco*RI site, and therefore contains chromosome 4-derived repeats and is sensitive to *Xap*I digestion. Patient 2, clinically diagnosed as possibly FSHD, was originally genetically confirmed as FSHD with a hybrid allele of 30 kb decreasing to 17 kb after *Eco*RI/*Bln*I

double digestion. Further analysis with *Xap*I revealed that the 30 kb fragment is a homogeneous 10-derived array based on its *Xap*I resistance. Consequently, the 17 kb *EcoRI*/BlnI fragment cannot be derived from the 30 kb array.

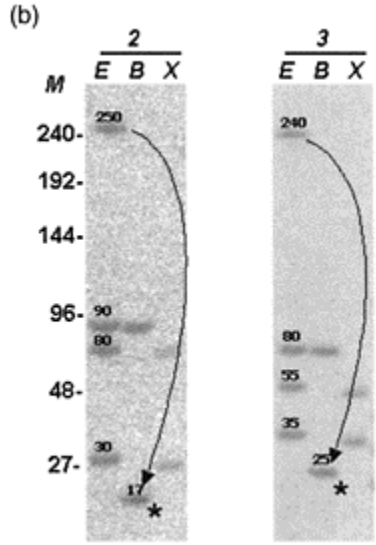

Figure 15.4b PFGE analysis of patient 2 carrying a potential hybrid repeat array of 30 kb. Combinatory analysis with *Eco*RI/*Hind*III, *Bln*I and *Xap*I shows that the 250 kb allele is the only allele that is sensitive to both discriminating enzymes. The other three alleles are homogeneous arrays since they are resistant to *Bln*I or *Xap*I. Therefore, it is concluded that the 17 kb *Eco*RI/*Bln*I hybrid fragment (asterisk) is not derived from the 30 kb allele, but rather from the 250 kb allele (arrow). FSHD is therefore excluded in this patient. Triple DNA analysis of

control individuals revealed another hybrid case (3) with a similar allele constitution. Marker sizes are indicated on the left.

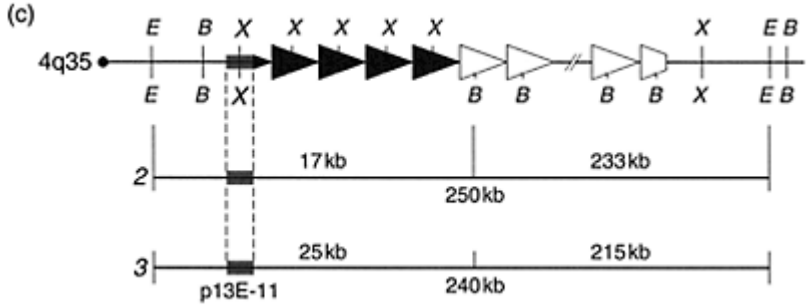

Figure 15.4c Schematic representation of the hybrid allele of patient 2 and individual 3. Chromosome 4-derived units are solid, 10-derived units are open. The restriction sites for *Eco*RI (E), *Bln*I (B) and *Xap*I (X) are indicated. Fragment sizes of the hybrid allele in patient 2 and individual 3 are indicated below the chromosome.

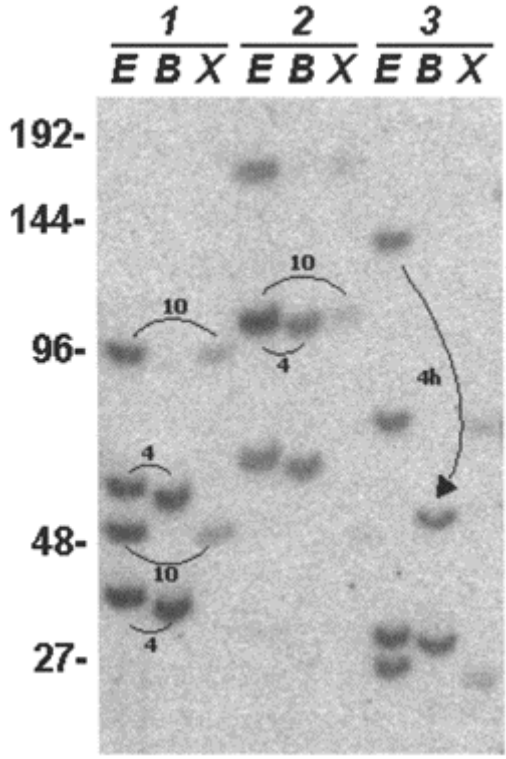

Figure 15.5 PFGE analysis of the D4Z4 repeat arrays on chromosomes 4 and 10 in three control individuals after digestion with *Eco*RI/*Hind*III (E), *Eco*RI/*Bln*I (B) or *Xap*I (X) and hybridization with probe p13E-11. Individual 1 carries a standard allele configuration of two 4-derived (*Bln*I-resistant) alleles of 65 kb and 40 kb, and two 10-derived (*Xap*I-resistant) alleles of 100 kb and 50 kb. Individual 2 carries co-migrating 4-derived and 10-derived alleles of 120 kb based on the resistance to both enzymes. Individual 3 has a hybrid allele of 140 kb, which is reduced to 55 kb (arrow) upon *Eco*RI/*Bln*I digestion and

subsequently has no corresponding 140 kb fragment in the *Xap*I lane. Marker sizes are indicated on the left.

clinical phenotype (Lemmers *et al.*, 2003). It is therefore important to use additional analytical tests for FSHD confirmation in the absence of a short 4q35-derived *Eco*RI fragment.

The most efficient way to detect extended deletions is by PFGE analysis on agarose-embedded DNA plugs (Lemmers *et al.*, 1998). All large-sized D4Z4 alleles are visualized and the absence of one allele is immediately suggestive of a D4F104S1 deletion. Unfortunately, all probes that recognize the D4Z4 repeat array proper show cross-hybridization in the FSHD range with many homologous regions and can therefore not be used for detecting the deleted fragment in *Eco*RI-digested DNA. Furthermore, since the proximal deletion breakpoint is not known, the observed fragment length will not provide any insight into the precise number of repeats borne by the D4F104S1 deletion allele. To circumvent this problem, one should perform a digestion with the frequent cutting enzyme *Mse*I (*Tru*91) (Cacurri *et al.*, 1998; Lemmers *et al.*, 1998, 2003). This enzyme recognizes the restriction site TTAA and therefore cuts on average every 100 bp, but not within the D4Z4 repeat array. By using this enzyme, all co-hybridizing DNA is digested in fragments smaller than 7 kb and does not interfere with the bigger D4Z4 repeat arrays. *Mse*I can be used, like *Eco*RI, in double digestions with *Bln*I to determine the chromosomal origin of the repeat arrays. Further, when using MseI, the number of D4Z4 repeat units can directly be derived from the repeat length by dividing by 3.3. A probe that recognizes the D4Z4 repeat unit itself (for example, 9B6A; Wright *et al.*, 1993) is used to visualize all four repeat arrays in *Mse*I-digested DNA *(Figure 15.6a)*.

FSHD diagnosis using linear gel electrophoresis often fails to separate all four D4Z4 alleles and therefore the absence of one allele, which is suggestive of a proximally extended D4Z4 deletion, remains undetected and may lead to a false-negative FSHD diagnosis. The strategy with *Mse*I as described in the PFGE section can also be used in a linear setting. This should allow even more accurate estimation of allele size and repeat number. The *Bgl*II/*Bln*I dosage test (Section 15.10; van der

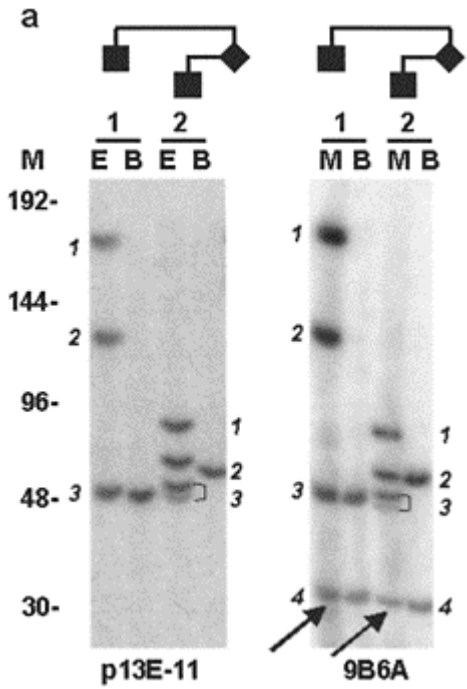

Figure 15.6a Pulsed-field gel electrophoresis (PFGE) analysis of two members of a family with a D4Z4 deletion including the probe region D4F104S1. Left panel: E *(Eco*RI/*Hind*III*)* and B (*Eco*RI/*Bln*I) digested DNA, hybridized with probe p13E-11. Affected individual 1 manifests three alleles with *Eco*RI, two 10-type alleles of 165 kb (1) and 130 kb (2) and one 4-type allele of 50 kb (3). Following *Eco*RI/*Bln*I restriction, only a 47 kb 4-type allele is visible. Molecular analysis of DNA from individual 2 also revealed the absence of a 4-type alleles since it visualizes only a 65 kb (2) 4-type allele, a 85 kb (1) 10-type allele and a mosaic 10-type allele (3) giving rise to fragments of 53

kb and 50 kb. Right panel: The D4F104S1 deletion allele can also be detected with paired M (*Mse*I, *Eco*RI) and B (*Mse*I, *Eco*RI, *Bln*I) digested DNAs after hybridization with 9B6A. This probe hybridizes to each D4Z4 repeat. An arrow indicates the deleted allele (4).

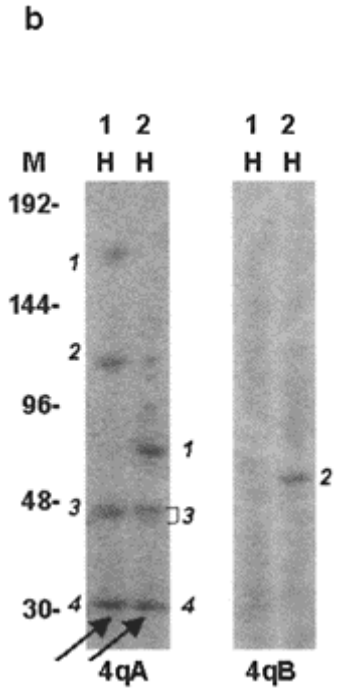

Figure 15.6b Hybridization of *Hind*III-digested DNA with distal probe 4qA (left) reveals the FSHD allele (4) with D4F104S1 deletion indicated with an arrow. Allele 2 of individual 2 has a B-type distal region and is vizualized upon 4qB hybridization of the same blot (right). This allele typing can also be used to

identify non-pathogenic 4qB-type alleles.

Maarel *et al.,* 1999) can help to detect D4F104S1 deletion alleles, but this test only indicates its presence and does not provide any clue as to the repeat length. Therefore, a new method was developed for detecting D4F104S1 deletions in the routine FSHD diagnostic setting, this involves the sequential use of several diagnostic probes on the same blot in which *Hind*III replaces *Eco*RI. Digestions with *Hind*III result in D4Z4 fragments which are 6 kb larger than those with *Eco*RI and allows one to use the probe 4qA in addition to p13E-1 1 that recognizes a sequence distal to the D4Z4 repeat array *(Figure 15.7)* (Lemmers *et al.,* 2002). Since this part of the disease allele is not deleted, this probe can be used to visualize these alleles. In this test, *Eco*RI, *Eco*RI/*Bln*I and *Hind*III-digested DNA is first hybridized with probe p13E-11, after which hybridization with probe 4qA visualizes the D4F104S1 deletion allele in the *Hind*III lane *(Figures 15.6b and 15.7).* Unfortunately, the exact

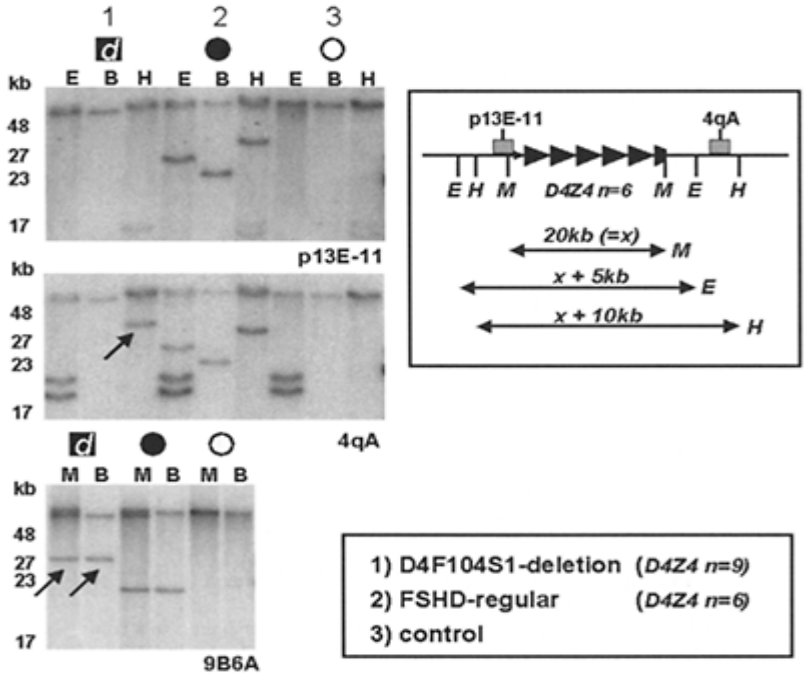

Figure 15.7 Use of probe 4qA on a standard conventional agarose gel to identify deletion junctional fragments in a D4F104S1-deletion family (*d*, sample 1). A FSHD case without p13E-11 deletion (sample 2) and a

healthy control (sample 3) are also shown ((genomic DNAs were digested with restriction enzymes E (*Eco*RI), B (*Bln*I) and H (*Hind*III). Southern blots were serially hybridized with p13E-11 and probe A)). Arrows indicate the p13E-11 deleted alleles. DNA digested with M (*Mse*I, *Eco*RI) and B (*Mse*I, *Eco*RI, *Bln*I), hybridized with 9B6A on a standard linear gel. This analysis allows the estimation of the exact number of repeats within the p13E-11 allele as well as the chromosomal origin. In the box on the right, a schematic presentation of the D4Z4 region on chromosome 4q35 is shown. Further the location of the *Eco*RI (E), *Hind*III (H) and *Mse*I (M) restriction sites adjacent to D4Z4 and probe p13E-11 is shown. Below the repeat array the expected length of the D4Z4 fragment of 6 units is depicted after digestion with *Mse*I, *Eco*RI and *Hind*III.

number of D4Z4 repeat units characterizing the FSHD-sized D4F104S1 deletion alleles as well as their chromosomal origin cannot be established by this strategy. This necessitates use of the *Mse*I method depicted in *Figure 15.7* and described in the PFGE section.

Only a few patients with proximally extended deletions have been described. However, the true frequency of proximally extended deletion is currently unknown, mostly due to the inability of the standard diagnostic setting to identify these cases. Yet many diagnostic labs describe apparent FSHD cases without a short FSHD size repeat. In such cases, we recommend the use of this methodology to detect D4F104S1 deletions. This will eventually allow the determination of the true frequency of these D4F104S1 deletions.

15.7 Short non-pathogenic alleles

Healthy control individuals normally carry D4Z4 fragments ranging from 41–335 kb, while patients have one chromosome 4-derived fragment of <38 kb. Nonetheless, several studies on the D4Z4 repeat length in different control populations revealed the presence of 4-type alleles of <38 kb (van Deutekom *et al.,* 1996; van Overveld *et al.,* 2000; Matsumura *et al.,* 2002). An inverse relationship has been established between D4Z4 repeat size and the severity and progression of FSHD (Lunt *et al.,* 1995; Tawil *et al.,* 1996; Ricci *et al.,* 1999), which might explain the reduced penetrance of arrays close to 38 kb in length. Furthermore, an estimated 1% of non-pathogenic cases can be explained by 4; 10 translocations in which the short 4-type D4Z4 resides on chromosome 10, rather than on chromosome 4. In other cases, short D4Z4 arrays in the control population could be explained by incorrect interpretation of the DNA analysis by the accidental co-migration of 10-type alleles and hybrid alleles as explained in Section 15.6. However, for some of these alleles, an explanation for their being non-pathogenic could not be given.

Recently, it has been shown that a region distal to the D4Z4 repeat array on chromosome 4 is polymorphic. Two distinct 4q alleles (4qA and 4qB) have been identified that differ by a few deletion and insertion events. The most prominent difference between 4qA and 4qB is the presence of a large beta-satellite repeat in the 4qA allele (van Geel *et al.,* 2002). Both alleles have been shown to be equally common in the population, but FSHD is exclusively associated with 4qA-type alleles (Lemmers *et al.,* 2002).

When DNA diagnosis of a FSHD patient reveals a FSHD-sized allele, one may assume that this is a pathogenic 4qA-type allele. FSHD has never been associated with short 4qB alleles and these alleles have recently been shown to be non-pathogenic (Lemmers, in preparation). It is however important to perform allelotyping in individuals carrying FSHD-sized chromosome 4 alleles without FSHD. Allelotyping can easily be performed on linear as well as PFGE/FIGE-separated *Hind*III-digested DNA with the sequential use of allele-specific probes 4qA and 4qB *(Figure 15.6b).*

All FSHD-sized 4-type alleles found in the Dutch control population were in the upper region of the FSHD size range. Allelotyping of these arrays (9–10 repeat units) revealed that they were equally distributed over 4qA and 4qB alleles (Lemmers, unpublished results). As described above, the short arrays on a 4qB allele could be excluded for FSHD. Since the D4Z4 arrays on 4qA alleles are all close to 38 kb in length, the penetrance of these alleles may be close to zero and are therefore unlikely to give rise to an FSHD phenotype.

15.8 Mosaicism in FSHD

In as many as 10–30% of FSHD cases, a *de novo* D4Z4 contraction is observed (Padberg *et al.,* 1995; Zatz *et al.,* 1995; Lunt, 1998). A high proportion of these new FSHD cases results from rearrangements probably early in embryogenesis resulting in somatic mosaicism for the disease allele in either patient or asymptomatic parent. Somatic mosaic patients have a smaller change of affected offspring than non-mosaic FSHD patients when the mosaicism is also present in the germ cells, since less than 50% of these cells carry an FSHD-sized allele. The possibility of genetic mosaicism has to be carefully

considered in FSHD families in the event of the recurrence of offspring with FSHD, or in those families suspected of manifesting anticipation (see Chapter 12).

A relationship has been established between the severity of the disease and a combination of (i) the percentage of blood cells carrying the disease allele and (ii) the D4Z4 repeat size of the disease allele (van der Maarel *et al.*, 2000). Patients in whom only a small proportion of blood cells carrying a FSHD-sized D4Z4 allele may still present with a FSHD phenotype. The suggestion that males are in general more severely affected than females (Lunt *et al.*, 1989; Padberg *et al.*, 1991, 1995; Zatz *et al.*, 1998) is especially significant in the case of somatic mosaicism. An excess of mosaic males over mosaic females is observed; since mosaic males already can have a moderate FSHD phenotype when only a small proportion of their cells carry a FSHD-sized allele. By contrast, females with a comparable proportion of affected cells and similar repeat size appear to be unaffected (van der Maarel *et al.*, 2000). Thus it is very important that somatic mosaicism is identified and the proportion of affected cells determined for predicting the progression of the disease.

Generally a mitotic D4Z4 repeat contraction on chromosome 4 gives rise to two cell populations, one carrying the original alleles prior to the rearrangement, and the other in which the D4Z4 repeat array of one of the 4qter alleles is rearranged to the FSHD size. The best way to detect mosaicism in *de novo* FSHD and to estimate the proportion of affected cells is by FIGE or PFGE preferably on agarose-embedded DNA plugs. As shown in *Figure 15.8* (right panel), PFGE analysis of DNA of a *de novo* FSHD patient reveals five alleles, indicative of somatic mosaicism: three equally strong hybridization signals of the non-mosaic alleles (one healthy chromosome 4 and two chromosome 10 alleles) and two less intense hybridization signals of the mosaic alleles (ancestral and *de novo* allele). If liquid DNA is used instead, one should be cautious of DNA shearing, which is especially clear for the alleles larger than 50 kb (*Figure 15.8*, individual 2 right panel). The proportion of affected cells in the blood can be estimated by comparing the intensity of the FSHD-sized allele with the mosaic ancestral allele, or when using liquid DNA with the intensity of alleles close to that of mosaic *de novo* allele.

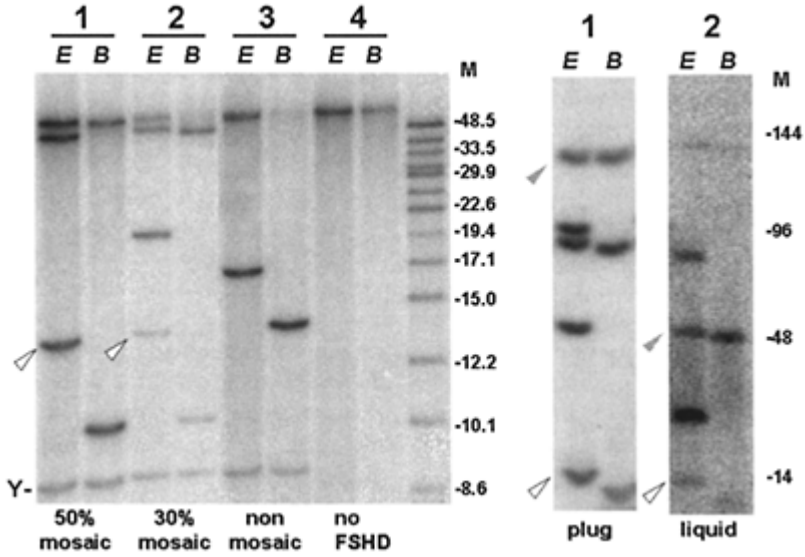

Figure 15.8 Southern blot analyses of *Eco*RI (E) and *Eco*RI/*Bln*I (B) digested DNA of mosaic (1 and 2), non-mosaic (3) and control (4) individuals and hybridization with probe p13E-11. The mosaic *de novo* FSHD alleles are marked with an open arrowhead, whilst the ancestral mosaic allele is marked with a closed arrowhead. A mosaic FSHD allele on linear gels can be recognized by a lower intensity of this *de novo* allele as compared to other non-mosaic alleles (sample 2). Comparison with the cross-hybridizing 9.4 kb chromosome Y fragment can also facilitate the identification of somatic mosaicism for FSHD. In general, PFGE analysis of a high-molecular-weight mosaic DNA sample reveals the presence of a fifth allele, three non-mosaic alleles in addition to the *de novo* and ancestral mosaic alleles (samples 1 and 2, right panel).

The use of agarose-embedded DNA plugs is superior to identify somatic mosaicism in FSHD. Since there is no DNA shearing, all non-mosaic alleles have 100% intensity and the proportion of mosaicism can be estimated from the ratio ancestral versus *de novo* mosaic allele (sample 1, right panel). The use of PFGE allows the identification of somatic mosaicism in as little as 5% of cells. Interestingly, individual 1 was originally not identified as a mosaic case using linear gel electrophoresis. Therefore, the proportion of sporadic mosaic patients may be underestimated with the use of linear electrophoresis only. Based upon the signal intensity of the *de novo* FSHD allele compared with other fragments, the level of somatic mosaicism is estimated to be 50% and 30% for individuals 1 and 2, respectively. Both individuals 1 and 2 have a 13.5 kb FSHD allele. Individual 3 has a 17 kb FSHD allele and is non-mosaic. For control individual 4, only alleles of >41 kb are visible. Marker sizes for linear gels are indicated on the right, whilst those of the PFGE gels are on the left.

Sometimes somatic mosaicism can also be detected with linear gel electrophoresis, most notably in mildly or unaffected parents of a *de novo* FSHD patient, with only a small proportion of the mosaic FSHD allele. With this standard technique, a weaker hybridization signal of the FSHD-sized allele compared to the signal of other alleles or co-hybridizing fragments in the same lane reveals somatic mosaicism for FSHD (*Figure 15.8*, individual 2, left panel). Comparing both electrophoresis techniques unambiguously shows that linear electrophoresis is not always capable of detecting somatic mosaicism. As shown for patient 1, the *de novo* FSHD allele is present in approximately 50% of the blood cells, which was only detected after performing PFGE analysis.

15.9 *Not*I-digest for chromosomal assignment

The chromosomal origin of D4Z4 repeat arrays is in general derived from the typing of D4Z4 repeats within the array based on *Eco*RI/*Bln*I and *Xap*I digestions. Owing to the occurrence of 4;10 translocations, this method is not completely reliable and sometimes requires the use of chromosome 4-specific probes for determining the chromosomal origin of the repeat array. Due to the high homology between 4q35 and 10q26 in the region from inverted repeat (D4S2463), 40 kb proximal of the D4Z4 repeat array, to the telomeric $TTAGGG_n$ repeats, 60 kb distal to the D4Z4 repeat, it is not possible to design a specific Southern blot probe within this region for one of these two chromosomes. Restriction analysis with *Not*I, releasing the most distal *Not*I fragment of chromosome 4q upon which D4Z4 resides *(Figure 15.1),* allows the use of a chromosome 4-specific probe. This probe only recognizes the 4q35 *Not*I fragment, which is usually 185 kb larger than the *Eco*RI fragment (Lemmers *et al.,* 1998). Probe p13E-11 recognizes the same fragments but also those of chromosome 10, which are about 300 kb larger than the *Eco*RI-fragment (Lemmers *et al.,* 1998). Comparing the lengths of the *Eco*RI fragments of the chromosome 4 and 10 alleles with the *Not*I fragments of the corresponding alleles unambiguously reveals *Eco*RI fragment derived from chromosome 4 and those from chromosome 10 *(Figure 15.9).*

This *Not*I digest requires the use of agarose-embedded DNA plugs (see Section 15.13) and PFGE (den Dunnen and van Ommen, 1993). Alongside chromosomal assignment of the D4Z4 repeat array in complex cases, this method is also recommended for determination of the size of a proximal deletion in patients with a D4F104S1 deletion (see Section 15.6).

15.10 *Bgl*II/*Bln*I dosage test

Diagnostic problems associated with 4; 10 translocations and deletions of the p13E-11 probe region require the use of PFGE/FIGE. Since most diagnostic centres lack PFGE/FIGE experience, the *Bgl*II/*Bln*I dosage test was developed to make use of conventional linear gel electrophoresis (van der Maarel *et al.,* 1999). This dosage test uses the *Bln*I polymorphism within the first repeat unit adjacent to p13E-11 to differentiate between 4-type and 10-type repeat units. It is therefore the first D4Z4 repeat unit that determines the type of the entire D4Z4 repeat array. Digestion with *Bgl*II releases a fragment of 4061 bp carrying p13E-11 and the first D4Z4 repeat unit. Subsequent digestion with *Bln*I will reduce the size of the 10-type fragment to 1774 bp, whilst the 4-type fragments remain undigested *(Figure 15.10a).* Following conventional gel electrophoresis and hybridization with p13E-11, the relative intensities of the two fragments can easily be scored by visual inspection or quantitative analysis programs and enables the identification of translocated alleles. In subjects without translocated alleles, the 4:10 ratio should be 1 (2:2), subjects carrying three 4-type repeats have a ratio 3 (3:1), while subjects carrying three 10-type repeats have a ratio of 0.33 (1:3) *(Figure 15.10b, c).* Since these 4;10 translocations have been shown to be very common in various control populations (van Deutekom *et al.,* 1996; Lemmers *et al.,* 1998; van Overveld *et al.,* 2000; Matsumura

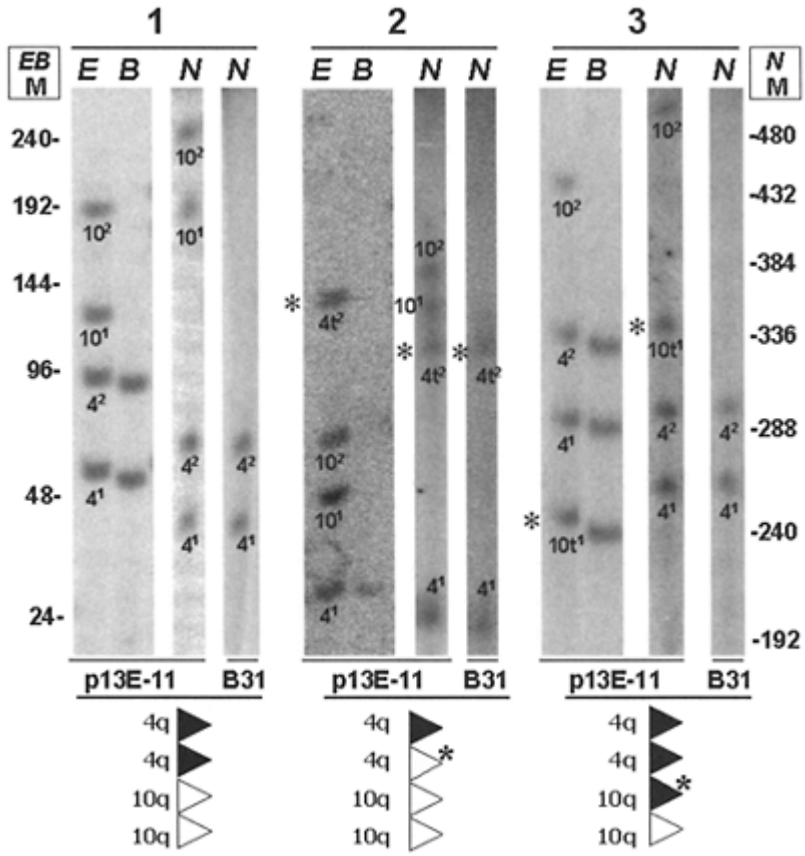

Figure 15.9 Allele assignment of the D4Z4 repeat array of three individuals by using *Not*I-digested DNA plugs. As shown in Figure 15.2, *Not*I digestion allows the use of chromosome 4-specific probes, like B31 (Lemmers et al., 2003). The *Not*I fragment on chromosome 4 is 185 kb larger than the *Eco*RI fragment on chromosome 4 and about 300 kb larger than the *Eco*RI fragment on chromosome 10. For each individual, three blots are shown, on the left a standard PFGE analysis with *Eco*RI/*Hind*III (E) and *Eco*RI/*Bln*I (B)-digested DNA hybridized with p13E-

11. In the middle and right, *Not*I-digested DNA of the same individual hybridized with p13E-11 (middle) and B31 (right). Standard PFGE analysis of individual 1 revealed no translocated alleles. Two chromosome 4 alleles of 55 kb and 95 kb are visualized at 240 kb (55+185) and 280 kb (95+185) upon *Not*I digestion and hybridization with B31. p13E-11 hybridization of the *Not*I blot show these chromosome 4 fragments in addition to chromosome 10 fragments of 425 kb (125+300) and 500 kb (200+300), corresponding to the 125 kb and 200 kb 10-type alleles in the *Eco*RI/*Hind*III and *Eco*RI/*Bln*I analysis. Individual 2 has three 10-type repeat arrays (50 kb, 70 kb and 135 kb) next to a 24 kb FSHD allele. *Not*I analysis shows that the 135 kb allele is translocated to chromosome 4 since it corresponds to a 320 kb (135+185) allele with probe B31. Other alleles are not translocated since the FSHD allele on chromosome 4 results in a 209 kb *Not*I fragment (24+185) and chromosome 10 alleles give rise to *Not*I fragments of 350 kb (50+300) and 370 kb (70+300). Individual 3 has three 4-type repeat arrays (40 kb, 75 kb and 110 kb) next to a 200 kb 10-type repeat array. Further analysis showed that the 40 kb repeat is translocated to chromosome 10 giving rise to a 340 kb (40+300) *Not*I fragment with p13E-11. The non-translocated 4 alleles are also visible with B31 hybridization and yield fragments of 260 kb (75+185) and 295 kb (110+185). The other 10-

type allele shows up at the expected 500 kb (200+300) after *Not*I digestion. D4Z4 allele constitutions are depicted below with filled (4-type repeat array) and open (10-type repeat array) triangles. The marker lane for the E/B blots is on the left (EB-M) and for N blots on the right (N-M).

et al., 2002) (see Section 15.4), also individuals carrying only chromosome 4-type D4Z4 alleles (4:0), as well as those carrying only 10-type alleles (0:4) have been detected. Finally, the *Bgl*II/*Bln*I dosage test also allows the detection of genotypes involving a D4F104S1 deletion (see Section 15.6). The *Bgl*II/*Bln*I dosage test provides insight into the reliability of the linear gel-based DNA diagnosis. Detection of the short chromosome 10-type allele in a FSHD patient with a 4:10 ratio of 1:3 is suggestive of a pathogenic hybrid allele. Furthermore, the short allele detected in an unaffected person with a dosage of 4:10 ratio of 3:1 is probably a 4-type array translocated to chromosome 10 and therefore non-pathogenic. Finally, a 4:10 of ratio 1:2 is suggestive of a D4F104S1 deletion (see Section 15.6). All these situations create a demand for additional diagnostic methods for reliable confirmation or exclusion.

15.11 Standard FSHD diagnostics

Molecular diagnosis for FSHD is used for diagnostic confirmation, presymptomatic carrier testing and for prenatal diagnosis. Subsequently samples are digested with *Eco*RI and, for 4/10 discrimination, with *Eco*RI/*Bln*I and with *Xap*I. Clinical diagnosis for FSHD is confirmed when a *Eco*RI fragment of <38 kb is detected with a corresponding 3 kb smaller *Eco*RI/*Bln*I fragment. *Xap*I is used to avoid possible incorrect FSHD interpretation in the presence of a co-migrating hybrid allele. The recombinogenic nature of the D4Z4 repeat array and the presence of 4;10 translocations sometimes necessitate additional digests (*Bgl*II/*Bln*I dosage test) and hybridizations for a reliable diagnosis, as has been described before.

In most diagnostic laboratories, linear electrophoresis is used to separate the FSHD allele (10–38 kb) from the larger alleles in a 40 hour run. Other laboratories use PFGE or FIGE, which takes about 20 hours using a specific program allowing separation of all D4Z4 fragments (10–350 kb) (van Deutekom *et al.*, 1996; Orrell *et al.*, 1999). Sizing of the D4Z4 fragments requires the use of an appropriate molecular weight marker. Markers ranging from 8.3 kb to 48.5 kb (Bio-Rad) are especially very useful for the accurate sizing of FSHD-sized alleles *(Figure 15.8)*. The sizing of alleles of >50 kb when using PFGE or FIGE requires the use of specific PFGE/FIGE markers (e.g. phage lambda concatamers).

There has been some debate about which electrophoretic technique, linear or FIGE/PFGE, is most suitable for routine FSHD diagnosis *(Figure 15.11)*. As discussed below, both techniques have their advantages and disadvantages. Linear electrophoresis

is still the most commonly used technique since it is part of the standard equipment in molecular diagnostic laboratories. Since it focuses on fragment sizes between 3 and 50 kb, it is very precise in sizing D4Z4 alleles in combination with a suitable molecular weight marker. Unfortunately, 4;10 translocations and the recombinogenic nature of the D4Z4 repeat sometimes complicate the FSHD diagnosis using linear electrophoresis. However, the introduction of 4/10 discriminating restriction enzymes *Bln*I and *Xap*I (see Sections 15.3, 15.5) and the *Bgl*II/*Bln*I dosage test (see Section 15.10) largely circumvents this shortcoming.

On the other hand, FIGE and PFGE permit the separation of all D4Z4 alleles and in combination with *Bln*I and *Xap*I, they provide complete allele information

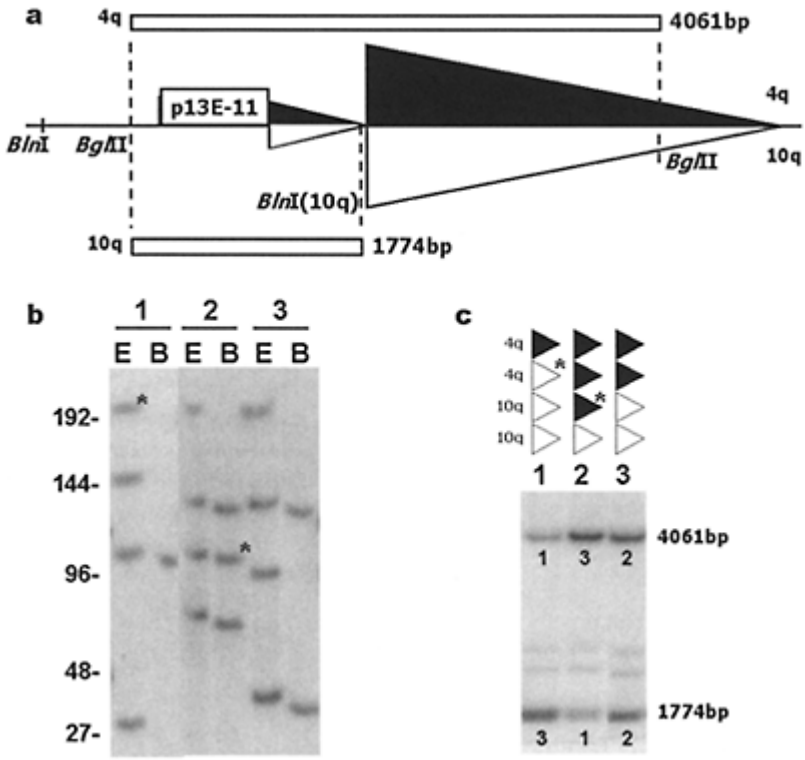

Figure 15.10 a. A schematic overview of the *Bgl*II/*Bln*I dosage test. DNA is digested with *Bgl*II and *Bln*I releasing the p13E-11 region including the first D4Z4 repeat array. Owing to the presence of a polymorphic *Bln*I site in the chromosome 10-type repeat unit, this analysis will release a 4061 bp

fragment from chromosome 4 and a 1774 bp fragment from chromosome 10 indicated by the bars respectively above and below the figure. b. PFGE analysis reveals the three most common D4Z4 repeat array constitutions. Sample 1 has three 10-type arrays (10% of Dutch population), sample 2 has three 4-type repeat arrays (10% of Dutch population). 4;10 translocations underlie these genotypes. Sample 3 has no translocated alleles and therefore two 4-type and two 10-type repeat arrays (80% of Dutch population). An asterisk indicates the translocated alleles. c. Dosage analysis of the three DNA samples depicted in (b). The chromosome 4- (4061bp) and 10- (1774) derived fragments are indicated with their respective chromosome numbers. Signal intensities from the fragments of both chromosomes (1:3, 3:1, and 2:2) can be compared to evaluate the presence of translocated D4Z4 alleles. Above the lanes, the repeat array constitutions of the different alleles can be seen. Filled triangles represent 4-type repeat arrays, while open triangles represent 10-type repeat arrays.

and can therefore elucidate almost all genotypes. Especially in individuals with a complex repeat array composition (see Sections 15.4, 15.5), in cases in which the probe region itself is deleted (Section 15.6), or where somatic mosaicism occurs (Section 15.8), PFGE has proven very useful (van Deutekom *et al.*, 1996; Lemmers *et al.*, 1998, 2001; van der Maarel *et al.*, 2000). Unfortunately, in most clinical molecular genetic or routine diagnostic laboratories, these techniques are not operational because there is a very limited need for this level of resolution and it

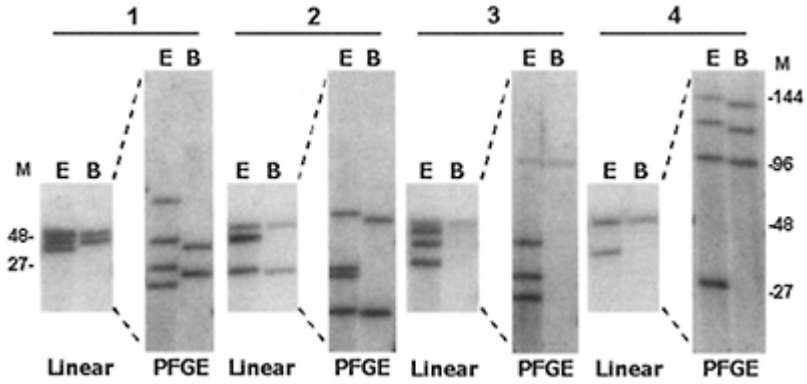

Figure 15.11 The superiority of PFGE/FIGE to linear electrophoresis in the separation of DNA fragments larger then 50 kb. *Eco*RI (E) and *Eco*RI/*Bln*I (B) digested DNAs of four individuals were separated by linear electrophoresis (left) and PFGE (right) gel electrophoresis. Individuals 1 and 2 do not carry translocated alleles in contrast to individuals 3 and 4. Marker sizes for linear gels are indicated on the left, whilst those of the PFGE gels are on the right.

requires specific equipment and expertise. Further, the wider separation range obtained using these techniques makes accurate sizing of FSHD-sized alleles more difficult.

15.12 DNA preparation

Since FSHD is associated with contractions in size of a large repeat array, and FSHD-sized alleles range from 10 to 38 kb, only Southern blotting-based techniques can be used for molecular diagnosis. Furthermore, high DNA quality is required as well as a relatively high DNA concentration for subsequent digestions. The isolation of liquid DNA by the 'salting out' procedure (Miller *et al.,* 1988) yields high-molecular-weight DNA that is suitable for this technique. Usually, with this method about 150 µg DNA can be isolated from 10 mL fresh blood. Prenatal diagnosis in the first trimester of pregnancy is performed on a chorionic villus biopsy sample (CVS), which in general provides sufficient high-molecular-weight DNA for FSHD testing. However, if amniotic fluid is

taken in the second trimester, the amniotic cells have to be cultured for 2–3 weeks to obtain sufficient cells for the isolation of the requisite amount of DNA.

Nowadays, many diagnostic laboratories opt to automate DNA isolation. For FSHD diagnosis, it is important to ensure that DNA quality and concentration is sufficiently high. This is especially important for the detection of somatic mosaicism, since it requires the comparison of signal intensities of the mosaic allele with other alleles in the same lane. Furthermore, visualization of the non- or partially separated alleles of >38 kb may serve as an internal control for the quality of the DNA in case no FSHD-sized fragments are detected *(Figure 15.8)*.

The recombinogenic nature of the repeat array and 4;10 translocation often requires the separation of all four alleles by means of PFGE or FIGE. The average length of *Eco*RI or *Hind*III-digested D4Z4-alleles is about 100 kb. Sometimes, the quality of DNA isolated by the 'salting out' procedure is sufficient to visualize all alleles. However, when these alleles are bigger than 100 kb or for the assignment of chromosomal origin (*Not*I, Section 15.9) the use of DNA in agarose plug is required (den Dunnen and van Ommen, 1993).

15.13 Specificity and sensitivity

The specificity of a diagnostic test is defined as the proportion of unaffected individuals who have a negative test. The sensitivity on the other hand is the proportion of affected individuals who have a positive test result. Since FSHD is characterized by a rather broad disease spectrum, clinical diagnosis of FSHD is very difficult and depends upon the expertise of the referring neurologist. Obviously, clinical misdiagnosis will dramatically influence the sensitivity of the DNA test.

The introduction of *Bln*I (see Section 15.3) into FSHD diagnostics significantly improved the sensitivity of the genetic test. In 1996, we reported the screening of 160 referrals in which a <38 kb 4-type allele was detected in 112 individuals (van Deutekom *et al.*, 1996) yielding a sensitivity of 64%. Lunt confirmed this sensitivity in a study of 270 patients in the UK (Lunt, 2000). In both studies a large proportion of the referrals without a short fragment were considered to be clinically unclear or referred for exclusion and therefore probably no FSHD. This results in a sensitivity level of the Dutch study of 90% and of the UK study of 94%. Conversely, other groups report sensitivities of 98–100% in clinically well-selected FSHD populations (Upadhyaya *et al.*, 1997; Orrell *et al.*, 1999). From the 676 cases which our group has analysed for FSHD, only 324 (48%) showed a fragment of <38 kb. This lower sensitivity can be explained by the fact that immediately after the introduction of DNA diagnosis, index cases probably had a very clear FSHD phenotype, whereas nowadays DNA diagnosis is often requested only for exclusion purposes for patients with only mild neuromuscular presentations. The 676 cases included a considerable number sent in for exclusion of FSHD. Including only cases with a classical FSHD phenotype sent in by an experienced neurologist for conformation of FSHD, a sensitivity of 95% is reached. It is to be expected that the introduction of *Xap*I (Section 15.5) and new methods for detecting D4F104S1 deletions (Section 15.6) will improve the detection of a FSHD-sized allele in patients.

The specificity of the DNA test has also increased dramatically since the introduction of *Bln*I. Short 10-derived D4Z4 fragments are observed in 11% of the control population (Orrell *et al.*, 1999; van Overveld *et al.*, 2000). The specificity of the DNA test, based on the short 4-type alleles found in the control population, varies between 94–100% (van Deutekom *et al.*, 1996; Orrell *et al.*, 1999; Lunt, 2000; van Overveld *et al.*, 2000). Moreover, these short alleles are in general in the upper region of the FSHD array size range. Allelotyping (see Section 15.7) and the introduction of *Xap*I will probably further increase the specificity of the test.

15.14 Concluding remarks

Over the last decade, molecular diagnosis of FSHD has been optimised, allowing diagnosis with an accuracy of >99%. Nevertheless, the complicated genetic rearrangement underlying FSHD necessitates a series of specialized DNA tests, all based on the laborious Southern blot technique. In *Figure 15.12* we present a flowchart of DNA diagnosis as performed in our laboratory. In the left panel, a relatively straightforward series of tests is presented to confirm FSHD as performed in the majority of cases. In the right panel, a more extensive scenario is drawn for complex cases.

Novel PCR-based diagnostic strategies failed due to the size of the residual repeat array that must be detected and the spreading of sequences homologous to D4Z4 over the entire genome while fluorescent *in situ* hybridization on metaphase chromosomes can only confirm the results obtained by Southern blot analysis. There is therefore still room for improvement in eventually replacing the laborious and technically difficult Southern blot strategy.

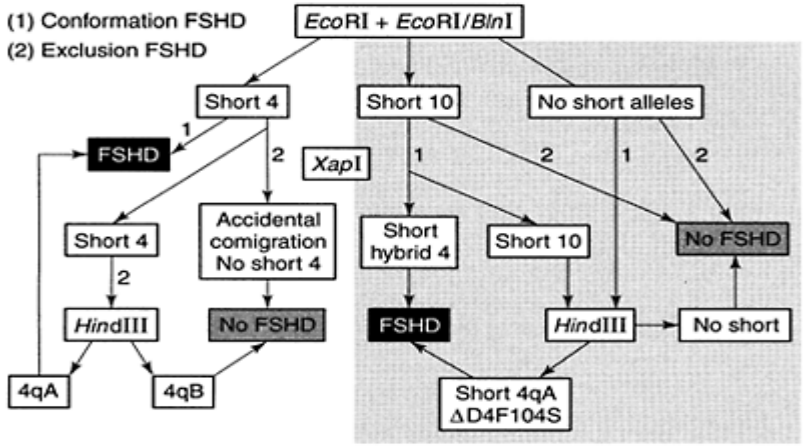

Figure 15.12 Flowchart for performing molecular diagnosis for FSHD for confirmation (1) as well as exclusion (2) purposes. The flowchart

starts with the regular *Eco*RI and *Eco*RI/*Bln*I digestions. When a short 4-type fragment is detected in DNA of a patient then FSHD is confirmed with >99% confidence. If a short 4-type allele is detected in an unaffected individual, *Xap*I digestion or allelotyping can be used to identify accidental co-migrating or non-pathogenic short 4qB alleles. The right panel of the scheme (shaded) can help to identify a FSHD allele in case the *Eco*RI and *Eco*RI/*Bln*I digestion did not reveal a homogeneous 4-type fragment in a patient. *Xap*I digestion can visualize hybrid FSHD alleles and by using *Hind*III and distal probe 4qA, or alternatively the *Bgl*II/*Bln*I dosage test, D4F104S1 deletions can be detected. The frequency of these complicated allele constitutions is currently unknown, but probably low.

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16.
FSHD myoblasts: *in vitro* studies

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

16.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common muscular dystrophy after Duchenne and myotonic dystrophy with an estimated prevalence of 1:20 000, resulting in severe disability in 15–20% of affected individuals. Clinical diagnosis of FSHD is based on the presence of a characteristic distribution of weakness in facial, proximal arm, and scapular musculature. EMG and muscle biopsy findings in FSHD are non-specific (Tawil and Griggs, 1997). FSHD is inherited in an autosomal dominant manner although as many as a third of cases have no family history and are the result of *de novo* mutations. The molecular rearrangements associated with the clinical phenotype of FSHD—deletions of integral numbers of 3.3 kb *Kpn*I units on chromosome 4q35 (van Deutekom *et al.,* 1993; Wijmenga *et al.,* 1994; Upadhyaya *et al.,* 1997), termed 'D4Z4 repeats', did not lead to the immediate identification of a single disease gene. Rather, extrapolating from studies in *Drosophila* and other organisms (Cattanach, 1974; Jaenisch *et al.,* 1981; Tartof *et al.,* 1984; Henikoff, 1994; Bedell *et al.,* 1996), the *position effect hypothesis* (Winokur *et al* 1994) proposed *cis*-interactions between the subtelomeric region of 4q35 and a gene (or genes) lying centromeric to the D4Z4 repeats. In this model, a dominant negative effect occurs as a result of the D4Z4 deletions in FSHD, possibly by derepression of a silent gene(s) or maintenance of a gene(s) in a transcriptionally active state after expression should have been down-regulated. Such a model is consistent with the autosomal dominant nature of the FSHD mutation. Subsequently, it was hypothesized that the unconventional coding sequence in each repeat (has been termed DUX4) may be expressed in FSHD, but not control, myoblasts (Gabriels *et al.,* 1999; Coppee *et al.,* 2002). Moreover, recent studies have demonstrated the presence of beta-satellite repeats downstream of the D4Z4 repeats on the allele which is deleted in FSHD (Lemmers *et al.,* 2002). This suggests that an additional parameter, perhaps related to locus-specific chromatin folding or binding of specific factors, plays a

role in the connection between the loss of D4Z4 repeats and the disease phenotype. While studies at the level of DNA sequence and chromatin organization on chromosome 4q35 proceed, we have pursued parallel studies *in vitro* to examine the effects of the molecular changes at the level of the muscle cell.

16.2 Myoblast morphology and replication

Examination of FSHD muscle pathology supports the conclusion that this is a primary myopathic disease, with neither primary neurogenic nor primary immune aetiologies. Therefore we believe that studies using skeletal muscle tissue should shed some light on the pathogenic process. In order to pursue our studies, we have utilized myoblast cell lines derived from FSHD patient and control muscle biopsies, and from other, non-FSHD, diseased muscle. The latter group includes muscle diseases such as Becker and limb girdle muscular dystrophies and nemaline rod, mitochondrial, vascular, congenital, and inflammatory myopathies. FSHD muscle biopsies are taken from muscles which will eventually become affected during the course of the disease; however, needle biopsy samples were taken from a patient's muscle which is still strong and not overtly dystrophic. Non-FSHD disease muscle biopsies were taken from the quadriceps. Normal muscle biopsies were taken from quadriceps or paraspinal muscles. All samples were taken with patient informed consent adhering to University Committee protocols on the use of human subjects.

16.2.1 Characterization of myoblasts

Myoblasts represent the daughter cells of activated stem cells which lie closely apposed to skeletal muscle fibers, and serve as a pool of myogenic precursors. Staining for desmin—an intermediate filament protein which is specifically expressed in myoblasts and not expressed in fibroblasts or adipocytes—confirms the high degree of purity attainable in human myoblast cultures. *(Figure 16.1)* Gene expression in myoblasts at different stages of differentiation is readily studied. Developmentally expressed genes which play a role in myoblast replication or in the onset of myogenesis may be up-regulated and down-regulated in a very short period of time. As an example, *Figure 16.2* illustrates the time course of expression of Id-1 protein in cultured, undifferentiated myoblasts over 5 days. Id-1 is a helix-loop-helix negative regulatory factor for myogenesis which is expressed only in undifferentiated myoblasts prior to their commitment to the myogenic program (Benezra *et al.*, 1990; Jen *et al.*, 1992; Wang *et al.*, 1992; Riechmann *et al.*, 1994).

16.2.2 Myoblast studies—cell biology

FSHD myoblasts display a necrotic phenotype (swollen, vacuolated cytoplasm) when maintained in normal cell culture growth conditions *(Figure 16.3)*, compared to normal myoblasts. This phenotype may be relatively specific to FSHD, since myoblasts affected by most other muscle diseases (disease controls) including myotonic dystrophy, Becker

myotonia and desmin storage myopathy, typically do not display a disproportionate number of necrotic cells.

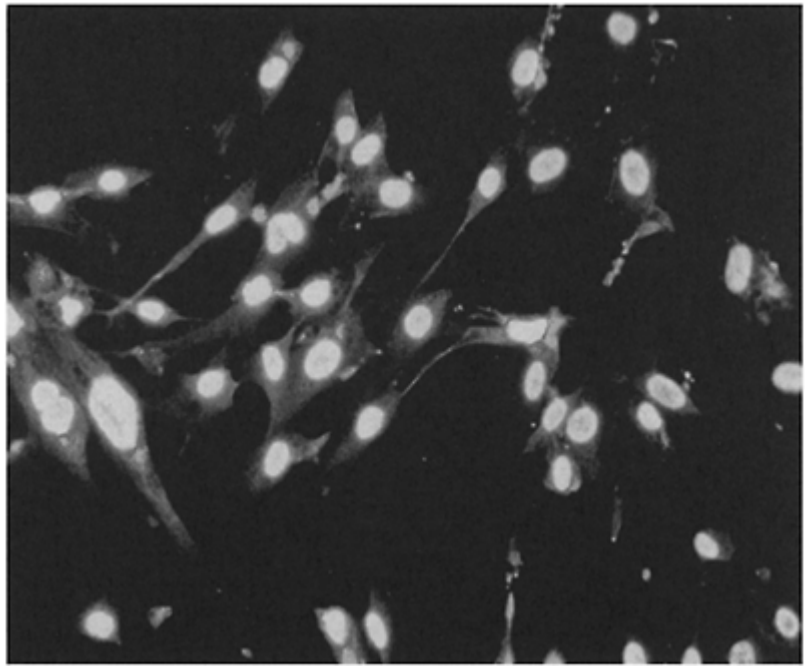

Figure 16.1 Immunostaining of SKMC normal muscle cell line with anti-desmin antibody. The homogeneity of myoblast cultures can be assessed by staining for desmin, an intermediate filament protein which is specifically expressed in myoblasts and not expressed in fibroblasts or adipocytes. The anti-desmin is detected with FITC- conjugated secondary antibody; a green reaction product would be found in the cytoplasm. As can be seen here, every cell in the culture has anti-desmin immunoreactivity. From this, we can conclude that we are preparing myoblast cultures of high purity.

16.3 Oxidative state

The potential contribution of oxidative free radical injury to muscle damage/necrosis in diseased muscle was studied in dystrophin-deficient myotubes and myoblasts isolated from the *mdx* mouse (Rando *et al.*, 1998). The authors identified a susceptibility to oxidative stress which was specific to the developmental stage of the muscle cells. While *mdx* undifferentiated myoblasts did not demonstrate an increased susceptibility to metabolic stressors as compared to controls, following onset of myogenesis the dystrophin-deficient myotubes exhibited an increased susceptibility to oxidative, but not other metabolic stress. This study demonstrated one form of downstream damage that could occur in *mdx* muscle cells, once the developmental stage for the primary genetic lesion had been reached.

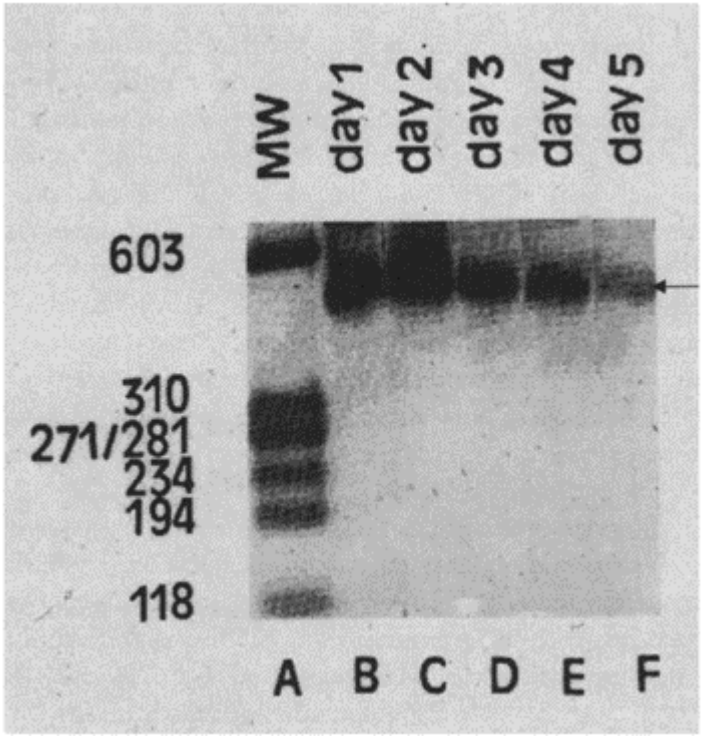

Figure 16.2 RT-PCR amplification of the Id-1 gene. RNA was isolated from myoblasts in culture. Each sample represents amplification of the Id-1 gene from an equivalent amount of mRNA. A—molecular weight marker, B—day 1, C—day 2, D—day 3, E—

day 4, F—day 5. Robust amplification of the gene transcript is observed from day 2 RNA. However, Id-1 mRNA are greatly decreased decrease by day 5.

16.3.1 Cell survival studies in FSHD myoblasts

In a similar fashion, we wished to investigate whether FSHD myoblasts might also demonstrate a developmental stage-specific vulnerability to oxidative, or other metabolic, stress. Myoblasts were subjected to oxidative stress using varying concentrations of paraquat, a generator of superoxide anion. Only very high concentrations of paraquat (20 mM) induced a mildly necrotic morphology in normal myoblasts. Conversely, low concentrations of this toxin (0.02 mM) exacerbated the morphologic phenotype of FSHD myoblasts. The muscle disease control cells demonstrated a mild response, if any (Winokur *et al.*, 2003). Cell survival studies were conducted under conditions of oxidative (paraquat) and non-oxidative (staurosporine, a protein kinase C inhibitor) stress. Myoblasts were allowed to grow to approximately 60% confluence, which typically required 24–48 hours. Skeletal muscle medium was removed and replaced with varying concentrations of medium containing toxins: 0.02 mM to 20 mM paraquat or 1 nM to 1 µM staurosporine. Adjacent control wells containing skeletal muscle media without paraquat or staurosporine corrected for the variation in growth rate among the different cell lines. The plates were incubated overnight and the number of surviving cells was obtained by counting. Survival was expressed as the number of live cells in

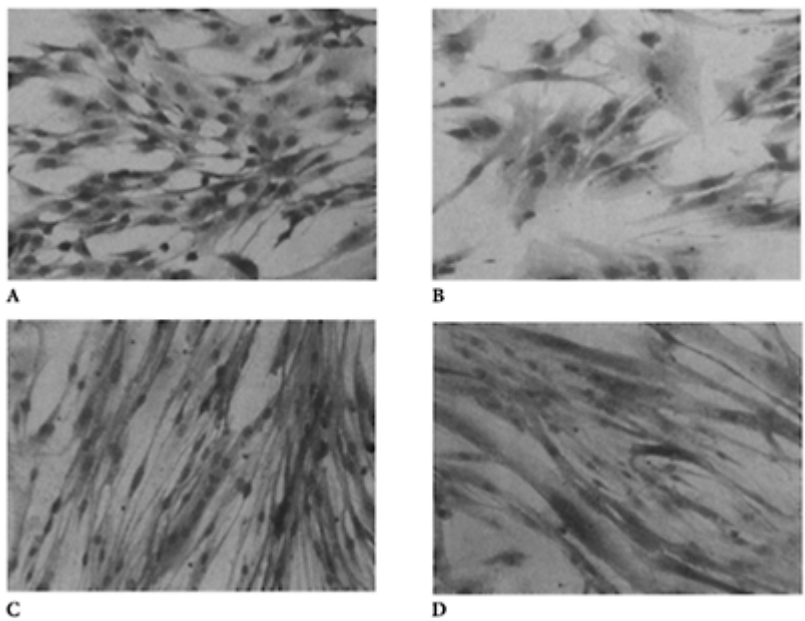

Figure 16.3 Wright-Giemsa staining of undifferentiated myoblasts: (A) control, (B) FSHD; following differentiation to form myotubes: (C) control, (D) FSHD.

test wells (containing toxin) relative to the number in the control wells for each cell line. Statistical significance was evaluated using a *t*-test. FSHD myoblasts, but not myotubes, were more vulnerable to paraquat than all controls for the lower concentrations of paraquat. No observed differences in susceptibility to staurosporine were noted for FSHD myoblasts relative to controls, regardless of differentiation status *(Figure 16.4).*

Our data demonstrate that one primary biochemical defect in FSHD appears at a very early stage of development, i.e. undifferentiated myocytes. A parallel analysis of comparative gene expression profiles between FSHD and control myoblasts revealed consistent down-regulation of a set of transcripts involved in oxidative stress, in FSHD myoblasts (Winokur *et al.*, 2003).

16.4 Question of cell cycle/premature differentiation/premature senescence

16.4.1 Intracellular redox state

A recent groundbreaking study (Smith *et al.*, 2000) compels us to consider the cellular redox state from a new perspective—not as the secondary consequence of other intracellular processes, but as an important gatekeeper in the determination of cell fate—the choice of replication vs. differentiation. In this study, the correlation between redox state and progression of cells to a differentiated state was exam-

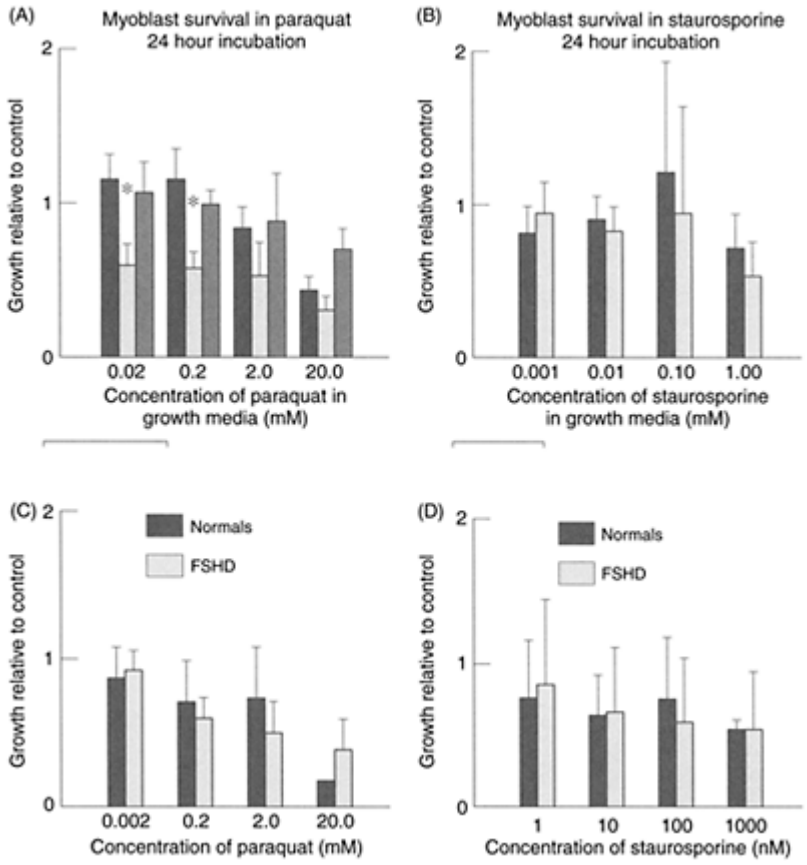

Figure 16.4 Myoblasts and myotubes exposed to paraquat and staurosporine. Myoblasts were exposed to toxins for 24 hours: paraquat (A) and staurosporine (B). The only statistically significant difference between FSHD cells and controls in susceptibility to toxin exposure was to low concentration (0.02 and 0.2 mM) paraquat. Myocytes were allowed to differentiate and were then exposed for 24 hours to paraquat (C) or staurosporine (D). There was no statistically significant difference between the survival of FSHD and

normal control myotubes exposed to either toxin.

ined in dividing oligodendrocyte-type-2 astrocyte progenitor cells. A correlation was established between the more oxidized state, leading to differentiation as opposed to a less oxidized state which was linked to continued replication. Reagents that altered the redox state of these cells directed them to the predicted fate—oxidizing agents increased differentiation whilst anti-oxidant strategies promoted cell division. Likewise, a correlation was established between the cell fate choice resulting from addition of a trophic factor or mitogen, and the direction of change of redox status of the cells (i.e. cells pushed to differentiate on addition of a trophic factor had become more oxidized, etc.). These data had direct implications for our studies of FSHD myoblasts, from which we have established that FSHD myoblasts are more susceptible to oxidative stress, are potentially more likely to leave the cell cycle, and resemble the senescent state of myoblasts derived from control muscle (see below). The ability to modulate the fate of FSHD myoblasts using trophic factors or other putative therapeutic agents may depend on manipulation of their intracellular redox state.

16.4.2 Senescence in FSHD myoblasts

We have observed that FSHD myoblasts have a morphologic appearance which is reminiscent of senescence in normal myoblasts *(Figure 16.5);* they subsequently differentiate to form myotubes which resemble those formed by senescent normal myoblasts *(Figures 16.3, 16.6).* It is important to note that many characteristics of FSHD myoblasts and myotubes are recapitulated by ageing normal cells *in vitro*. Of even greater significance is the fact that FSHD myoblasts display a decreased replicative capacity reminiscent of senescence in normal myoblasts *(Figure 16.7).*

16.4.3 Cell cycle dysregulation/p21 detection in FSHD—a connection with oxidative stress?

Oxidative stress of fibroblasts induces the expression of the cyclin-dependent kinase (cdk) inhibitor, p21 (Yin *et al.*, 1999). Since our cell biology and GeneChip

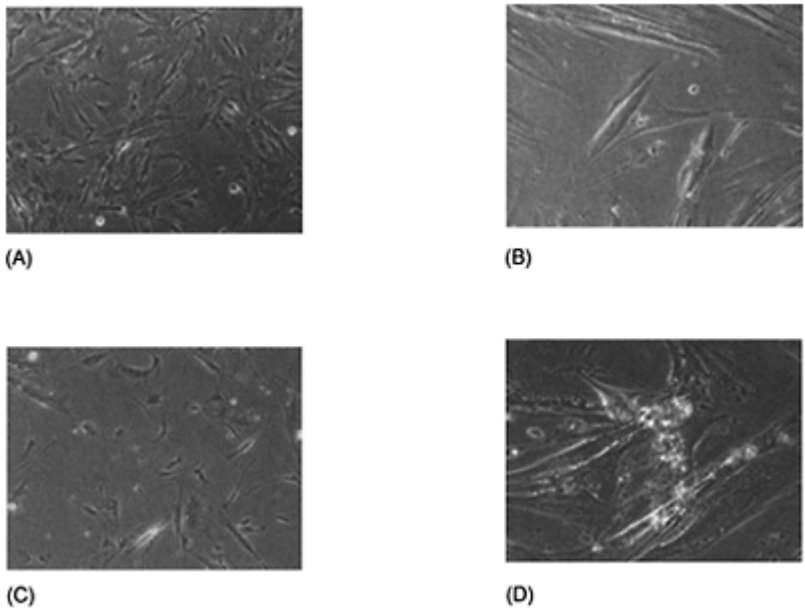

Figure 16.5 Morphological changes in normal and FSHD myoblasts aged *in vitro*. An increased oxidative state may be attributable to senescence. Several preliminary indicators were evaluated to determine the proximity of FSHD cells to senescence. First, normal cells were aged *in vitro* and their morphology was compared to that of FSHD cells without *in vitro* aging. The swollen, 'necrotic' phenotype predominates in normal myoblasts aged *in vitro*.

(A) Normal myoblasts during an early passage (not aged in vitro). Cells contain little cytoplasm and few, if any, vacuoles. 10×. (B) Normal myoblasts in senescent cell culture. Cells are swollen and vacuolated compared to myoblasts of earlier replicative age (A). These cells are not

as bizarre appearing as FSHD myoblasts artificially senesced in the laboratory. 20×. (C) FSHD myoblasts during an early passage. Although they are not aged in vitro, many cells are swollen and vacuolated. 10×. (D) FSHD myoblasts in senescent cell culture. Note the bizarre, swollen and vacuolated morphology which is exaggerated compared to cells of lower replicative age (C). 20×.

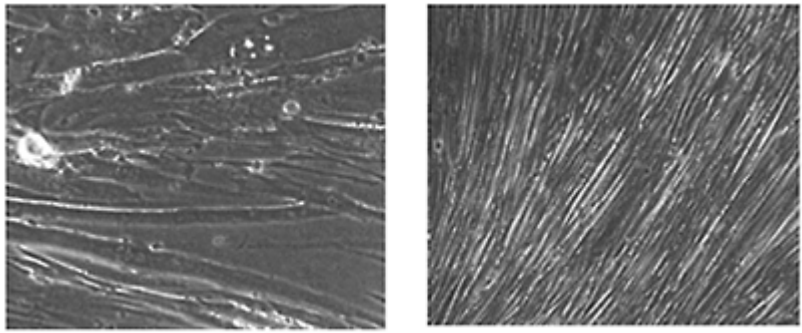

Figure 16.6 Differentiation of aged, normal myoblasts. The aged normal cells differentiate, even under conditions conducive to growth, to form myotubes. The resultant myotubes are somewhat swollen and disorganized and thus resemble FSHD myotubes that were not aged *in vitro*. The morphology of aged myotubes may result from the lack of confluence of the preceding myoblasts, or may involve some other feature of cellular senescence. (A) Control myotubes in a senescent cell culture exposed only to skeletal muscle growth medium (SkGM) containing 16.6% fetal bovine

serum. The majority of senescent myoblasts differentiated during exposure to growth factors (20X). (B) Low-magnification photo of highly confluent, non-aged control myotubes. Cells are highly organized. No similar field was ever found in FSHD cultures (10X).

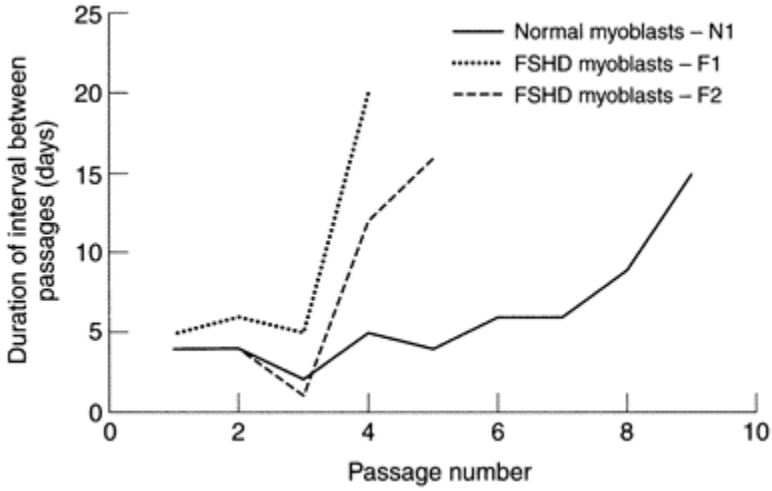

Figure 16.7 *In vitro* replicative history for FSHD and normal myoblast cell cultures. Although it is impossible to determine the *in vivo* replicative history of human cells, some indication of generational age was attained by measuring the number of passages and interval between passages for some myoblasts used in our preliminary studies. As cells approach senescence, their proliferative rate decreases, thus the time between passages should increase. The number of passages until senescence should be low relative to 'younger' cells. This figure shows the *in vitro* replicative history for two

FSHD and one normal cell culture. The number of passages for the FSHD cultures is considerably lower than for the normal cells. Likewise, the duration between passages is longer for the FSHD myoblasts. This suggests that the FSHD cells are closer to senescence. All cells were evaluated from the time they were thawed. Therefore, either cell aging occurred *in vivo,* or FSHD satellite cells have become primed for senescence once they proliferate in culture.

studies focused our attention on genes related to oxidative stress and cell cycle, we have investigated whether p21 is up-regulated in FSHD vs. control myoblasts under normal growth conditions. Protein expression by myocytes was evaluated using immunocytochemistry with rabbit p-21 antiserum (Santa Cruz Biotechnology). Signal was quantified by capturing images of several fields within each cell line/condition, and setting a threshold for positively labelled cells, using ImagePro. Nuclei staining positively for p21 are expressed as a percentage of all nuclei in the field: 13.2±2.0 for normals vs. 22.2±2.0 for FSHD, P=0.007. These results suggest that *even under normal growth conditions,* FSHD cells express higher levels of p21 than normal control myoblasts *(Figure 16.8).*

Progression of the cell cycle from the growth-factor-responsive stage (G1) to the S phase of DNA synthesis represents a tightly regulated checkpoint. p21 is an inhibitor of the cyclin-dependent kinases (cdks) needed for this progression (Cook *et al.,* 2000); up-regulation of p21 leads to exit from the cell cycle. Several pathways are known to be involved in the up-regulation of p21; for example, oxidative stress of fibroblasts induces the expression of p21 in a p53-dependent manner (Yin *et al.,* 1999). (p53 is a major mediator of intracellular pathways related to DNA damage or cell stressors.) Up-regulation of p21 followed by exit from the cell cycle and myoblast differentiation have been shown to lie downstream of up-regulation of the myogenic regulatory factor MyoD (Guo *et al.,* 1995; Halevy *et al.,* 1995; Otten *et al.,* 1997; Puri *et al.,* 1997a) although the two are not absolutely coupled (Parker *et al.,* 1995; Puri *et al.,* 1997b; Sabourin *et al.,* 1999). In the absence of MyoD, the myogenic program is delayed and significantly diminished, but is still observed,

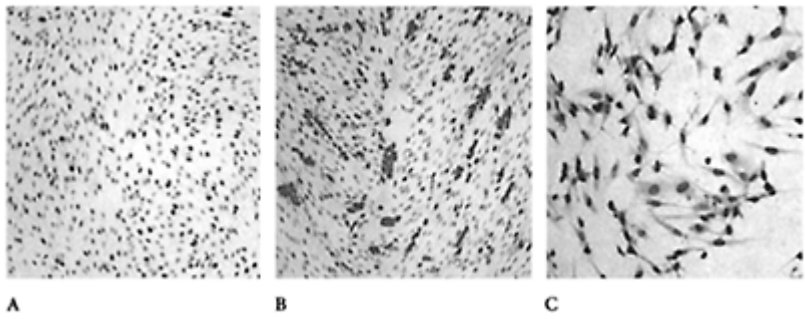

Figure 16.8 Immunocytochemical staining for p21 in (A) normal myoblasts, (B) partially differentiated myocytes and (C) FSHD myoblasts. Cells were grown in skeletal muscle growth medium then transferred to differentiation medium (B only) for 4 days. In the undifferentiated state (A, C), three distinct subpopulations of cells exist with regard to nuclear immunostaining for p21: heavily stained, intermediate, and lightly stained (negative for p21). The heavily stained cells may be targeted for differentiation. Once differentiation commences, the nuclei in fused cells (B) are exclusively positively stained for p21. The majority of unfused cells contained negatively stained nuclei. A large proportion of cells in the FSHD culture (C) are positively stained for p21 relative to the control (A).

perhaps due to some functional redundancy with the myogenic regulatory factor (MRF) Myf5 (Rudnicki *et al.,* 1992; Cooper *et al.,* 1999; Sabourin *et al.,* 1999; Yablonka-Reuveni *et al.,* 1999) or other factors not yet identified. p21 up-regulation in the absence of MRFs results in a reversible state of cell cycle arrest for myoblasts (Puri *et al.,* 1997b); it is possible that myoblasts in this quiescent state may be increasingly susceptible to cell death as they are not able to differentiate.

Since p21 is up-regulated in FSHD myoblasts, it is important to know whether this occurs in conjunction with up-regulation of the transcription factor MyoD and entry into

the myogenic programme (as observed in normal muscle differentiation), or whether it occurs independently of MyoD. Normal myoblasts expressing high levels of p21 were found to be targeted for differentiation *(Figure 16.8B)*. This correlation was not seen in FSHD myoblasts—p21 is robustly expressed even in cells which are not aligned or confluent *(Figure 16.8C)*. Entry into myogenesis was evaluated by quantifying the fusion rate for normal and FSHD cultures at selected time points after the exposure of cells to conditions favouring differentiation. FSHD myocytes fuse at a faster rate than controls, suggesting that despite their morphological appearance in the undifferentiated state, the muscle differentiation programme has already been turned on *(Figure 16.9)*. Taken together, these data suggest that the up-regulation of p21 in FSHD myoblasts is likely to represent, at least in part, withdrawal from the cell cycle linked to the Myo-D induced myogenic programme.

Currently we are investigating whether the p53-mediated cell cycle checkpoint may also contribute to the up-regulation of p21 in FSHD myoblasts. This pathway has recently been implicated in the progression of MyoD-induced myoblast differentiation (Puri *et al.*, 2002; Wang *et al.*, 2002). Puri *et al.* (2002) describe a phenotype for DNA-damaged C2C12 myoblasts which resembles what we have observed *in vitro* with FSHD myoblasts. The authors suggest that these abnormal C2C12

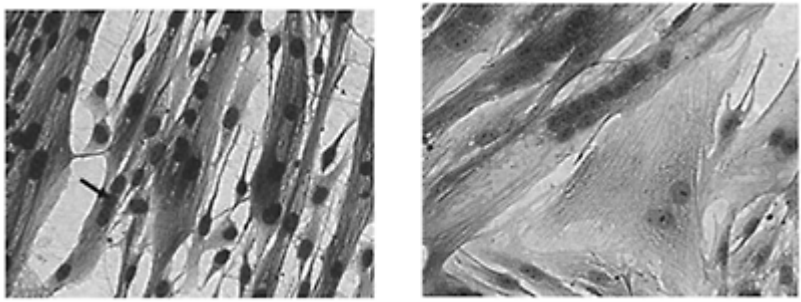

Figure 16.9 Fusion rate in FSHD and control myocytes. A representative field from a culture of (A) normal myocytes and (B) FSHD myocytes after 96 hours in differentiation medium (2% horse serum in DMEM). A mean of 83.5% of normal myonuclei had fused (a fused nucleus is depicted by arrow) to form myotubes at this time point while 93.1% of FSHD myonuclei had fused to form swollen, disorganized myotubes at this time point. Wright-Giemsa stain (40X).

muscle cells may result from altered differentiation after over-riding the DNA-damage-mediated checkpoint arrest. Our observations thus far lead us to conclude that FSHD myoblasts fuse and enter the myogenic programme prematurely. Much is now known about the chronology of gene expression in the various stages of normal myoblast differentiation (Bergstrom *et al.,* 2002); a comparable analysis of myogenesis in differentiating FSHD myoblasts will reveal precisely how this gene expression programme varies in FSHD myogenesis.

16.4.4 Senescence in FSHD myoblasts—replicative or premature?

Normal senescence in a cell population involves the progressive shortening of the telomeres with each round of replication, hence the description 'replicative senescence'. After a certain number of cell divisions, a cell will be withdrawn from the cell cycle. Termination of cellular replicative capacity is believed to be mediated via the activation of p53, followed by induction of the cyclin-dependent kinase (cdk) inhibitor p21. This represents the major signalling pathway linking reduced telomere size and cell cycle arrest. Progressive telomeric shortening has been correlated with the number of cell divisions in fibroblasts (Allsop *et al.,* 1992) and in muscle satellite cells (Decary *et al.,* 1997). The change in telomere length per cell population doubling, or telomere shortening rate, has been shown to vary in fibroblasts in relation to oxidative stress (vonZglinicki *et al.,* 1995; Martens *et al.,* 1998; Serra *et al.,* 2000). In one study of human fibroblast cultures, there was a reciprocal correlation between the change in telomere length per population doubling and the activities of two antioxidant enzymes—glutathione peroxidase and Cu/Zn super-oxide dismutase; in another study, treatment of fibroblasts with the free radical scavenger slowed the telomere shortening rate. Moreover, it has been shown that brief exposure (5 hours) of human fibroblasts to sublethal levels of the oxidative stressor tert-butylhydroperoxide followed by 2 days of recovery nonetheless led to a loss of replicative capacity equivalent to naturally aged fibroblasts (Dumont *et al.,* 2000). In addition to up-regulation of p21, Dumont *et al.* (2000) report the increased expression of genes associated with senescence (for example fibronectin, osteonectin, etc.) Despite the brief nature of the stress, the appearance of these biomarkers of senescence may be irreversible. It has been further speculated that accumulation of such stress-induced senescent cells in a given tissue may either alter tissue function or modify some properties of adjacent cells. This phenomenon of stress-induced senescence is one type of premature senescence, and reflects a loss of replicative capacity that is not related to withdrawal from the cell cycle triggered by the number of cell divisions.

FSHD myoblasts have a morphological appearance and a decreased replicative capacity reminiscent of senescence in normal myoblasts. Morphologic changes, elevated p21 expression, and vulnerability to oxidative stress can all be attributed to advanced replicative age. Our studies established that FSHD myoblasts are dividing less robustly—either because they are leaving the cell cycle prematurely, or because they are displaying replicative senescence. Because we wished to establish whether FSHD myoblasts have experienced true replicative senescence, or whether their loss of replicative capacity is based on other mechanisms disrupting the normal cell cycle, cultures of normal, FSHD, and muscle disease control myoblasts have been evaluated for replicative age using a telomere content assay. We have quantitated centromeric and telomeric DNA of myoblast

samples from FSHD patients, muscle disease controls and normal controls. The T/C ratio would be expected to be significantly less in FSHD myoblasts if muscle satellite cells had undergone a greater number of divisions prior to muscle biopsy. However, our data reveal no difference between FSHD and control myoblasts (Figlewicz *et al.*, 2002).

16.5 Discussion/future directions

In summary, our observations of FSHD myoblasts suggest several possible pathways to investigate. Our data suggest that the myoblasts have a constitutively altered redox state; this may or may not be alterable by antioxidant or trophic factor therapies. The FSHD myoblasts also have a reduced replicative capacity and a morphological appearance resembling senescence. Either of these two conditions (chronic oxidative stress or senescence) may be the cause of the up-regulation of p21 we have observed. In the case of such intracellular stress, p21 is activated secondary to up-regulation of p53. Alternatively, p21 may be elevated subsequent to activation of MyoD—the normal pathway of events in myogenesis. We are now investigating these hypothetical mechanisms.

One may ask why it is important to study myoblasts when FSHD is a disease of mature skeletal muscle. There are several responses to this very valid question. First of all, the pathologic changes in dystrophic muscle of FSHD are non-specific and do not point to any discrete uniform pathologic process, such as a storage disease. However, the dystrophic processes in mature muscle may also be altering properties of the satellite cells that reside there. One study comparing myoblasts from several stages of muscle development *in vivo* identified the differential expression of genes that play a role in the signalling pathways downstream of a growth factor (i.e. at a certain stage of development, the resident satellite cells/myoblasts may or may not be responsive to the growth factor, depending on such gene expression) (Zapelli *et al.*, 1996). In another study, myoblasts isolated from adult skeletal muscle transiently expressed some genes associated with adult skeletal muscle before displaying a pattern more characteristic of the undifferentiated state (Gunning *et al.*, 1987). These studies both provide evidence which suggests that all myoblasts are *not* equal—not only is there possible variation in their pattern of gene expression with respect to differentiation, but their intrinsic responsiveness to trophic factors or mitogens may be determined by their interaction with the skeletal muscle in which the satellite cells reside. An early observation, similar to ours for FSHD myoblasts, was the report of a significant loss of replicative capacity in myoblasts isolated from Duchenne muscle (Blau *et al.*, 1983). This defect could not be linked to the Duchenne locus on the X chromosome (Webster *et al.*, 1986), and when the dystrophin gene *(DMD)* was identified, it was hypothesized that the loss of replicative capacity of Duchenne myoblasts represented the senescence of the satellite cell population after years of intense skeletal muscle degeneration and regeneration. However, this hypothesis must also be abandoned, in the light of data which demonstrate (using a different culture technique) that many more myoblasts and satellite cells are available in the defective muscle fibres than give rise to clones when using the conventional myoblast culture techniques (Bockhold *et al.*, 1998). These authors conclude, ‘whatever the specific mechanism underlying ineffective muscle

regeneration…*it seems best explained in terms of a poor myogenic environment generated by the chronic progression of the disease rather than as loss of myogenic potential* per se'. This interpretation might also be valid with respect to our findings in FSHD myoblasts.

In recent years, investigations of FSHD have led to the characterization of the DNA sequence, identification of genes and pseudogenes located on chromosome 4q35; more detailed knowledge of the range of possible mutations on 4q35, the nature of the recombination events involving chromosome 10q26, and the relationship between genotype and clinical phenotype; changes both in global gene expression and in the expression of 4q35-specific genes in FSHD patient muscle biopsy samples; organization of the D4Z4 chromatin with respect to methylation and DNA-binding components; and the characterization of a new family of genes containing a double homeobox—the DUX genes—whose chromosome 4 copies are found within the D4Z4 repeats. How do our results with FSHD myoblasts fit into this complex picture of altered DNA sequence, altered chromatin, altered gene expression and specific clinical phenotype? One connection may come from the recent identification of a multiprotein complex which binds D4Z4 (Gabellini *et al.*, 2002); this complex includes YY1 (Yin Yang 1), a homologue of the *Drosophila* polycomb gene *pho*. Although polycomb gene group proteins were originally studied for their role in the long-term maintenance of gene silencing, studies in recent years have implicated them in a number of other important roles including control of cell proliferation. YY1 is a multifunctional transcription factor with known roles in gene activation and repression. In rodent myoblasts, YY1 protein is down-regulated during differentiation as a result of post-translational mechanisms (Walowitz *et al.*, 1998) suggesting not only a relationship with the myoblast cell cycle, but also that the key role of YY1 manifests at an early developmental stage in muscle. The link between YY1 binding at D4Z4, *cis*-regulatory interactions with DUX 4 or other 4q35 genes, *trans*-activation of other genes, and modulation of the myoblast cell cycle remains to be determined.

Lastly, we wish to consider the connection between our results and the development of therapy for FSHD. This autosomal dominant disorder may not simply be the result of the overexpression of a single aberrant protein, but is more likely to be the result of the complex dysregulation of a number of proteins. Which of these dysregulated proteins may be chosen for therapeutic manipulation remains to be seen. However, it is becoming increasingly likely that therapy for human muscle disease may be mediated via genetically modified myoblasts or muscle-derived stem cell infusions (Blau and Springer, 1995; Gussoni *et al.*, 1999; Seale and Rudnicki, 2000). As research of these therapeutic techniques progresses, an important consideration will be the viability and replicative capacity of the transplanted cells once they have become established adjacent to muscle fibres.

Hence the long-term efficacy of such therapeutic approaches will depend on the understanding of the effects which this specific muscle disease has on resident populations of muscle stem cells, and the myoblasts arising from them.

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17.
Exploring hypotheses about the molecular aetiology of FSHD: loss of heterochromatin spreading and other long-range interaction models

Melanie Ehrlich

FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

17.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is a unique genetic disease in that its molecular aetiology involves a shortening of an array of large tandem repeats on only one homologue at the disease-associated locus. This locus lies within the subtelomeric region of the long arm of chromosome 4 (Chr4), and the repeat is a 3.3 kb sequence termed D4Z4. Although the immediate genetic defect in FSHD has been identified, the way in which it causes the characteristic type of muscular dysfunction (Lunt, 2000) is still mysterious. In almost all FSHD patients there are between one and ten tandem copies of the D4Z4 repeat at the 4q35 band of one Chr4 homologue (Lemmers *et al.,* 2001). By contrast, unaffected individuals have 11–100 copies of this copy-number polymorphic repeat on both Chr4 homologues.

In this chapter, disease-associated 4q35 arrays of D4Z4 repeats with fewer than 11 copies will be referred to as short arrays. The most frequently invoked hypothesis for how short D4Z4 arrays on one 4q35 allele lead to the FSHD syndrome involves the assumption that the long D4Z4 arrays are normally heterochromatic and this heterochromatinization in unaffected individuals spreads from the array to genes important to FSHD on 4q35 (Hewitt *et al.,* 1994; Winokur *et al.,* 1994; Lee *et al.,* 1995; Gabellini *et al.,* 2002). It has been proposed that this putative normal heterochromatinization at 4q35 is lost when there is only a short D4Z4 array on one Chr4 homologue. The consequence of loss of this heterochromatin spreading would be an inappropriate increase in expression of critical genes in *cis* in affected skeletal muscle.

The hypothesis of loss of normal heterochromatin spreading at 4q35 in FSHD patients was derived from a type of *cis*-regulated spreading of gene repression called position effect variegation (PEV) (Weiler and Wakimoto, 1995; Wakimoto, 1998; Talbert and Henikoff, 2000). PEV usually results from genes being placed in proximity to constitutive heterochromatin, such as centromeric heterochromatin. It has been studied most thoroughly in *Drosophila*. In the case of FSHD, such *cis*-spreading of repressive heterochromatin structures has been proposed to display a gradient effect with genes closer to the normal, long D4Z4 arrays more affected than more distant genes (Gabellini *et al.,* 2002). I will refer to this type of *cis*-spreading of repressive structures as the loss-of-PEV hypothesis. The application of this hypothesis to FSHD is predicated upon the loss of genetically programmed repression rather than the gain of repression, as is usually the case for PEV. In PEV there is a decrease in the percentage of cells expressing a euchromatic gene because it has been inserted in the vicinity of heterochromatin by a rearrangement (Pirrotta and Rastelli, 1994). Once repression of the translocated gene is established, it tends to be inherited by progeny cells, but the whole cell population exhibits mosaicism (variegation) due to stochastic events determining which cells with identical DNA rearrangements will be subject to the repression emanating from the translocated gene. PEV can also result from multimerization of a DNA sequence, for example, 3–7 copies of a 10 kb *Drosophila* transposon, which presumably serves to confer a heterochromatic structure (Dorer and Henikoff, 1997; Talbert and Henikoff, 2000). In the case of PEV, that is due to the proximity of the gene of interest to a tandemly repeated sequence, the severity of the PEV-induced silencing correlates with the repeat copy-number somewhat analogous to the PEV effect proposed for D4Z4 arrays. PEV-like *cis*-acting repression due to a gene's proximity to heterochromatin has been assumed to display a gradient of repression along the DNA because of a gradient of spreading of chromatin condensation (heterochromatinization) from the heterochromatin. However, as described below, PEV need not show such a gradient. Mammalian cells also exhibit PEV-like effects including repeat-induced gene silencing (Garrick *et al.,* 1998; Festenstein *et al.,* 1999; McBurney *et al.,* 2002). Nonetheless, experimentally induced juxtaposition of constitutive heterochromatin and gene regions sometimes does not affect transcription of those genes (Bayne *et al.,* 1994) and does not cause spreading of heterochromatinization (Nicol and Jeppesen, 1996).

Telomere silencing at broken chromosome ends (Pryde and Louis, 1999) in yeast is the best understood, and apparently the simplest, of the PEV-related phenomena (Hecht *et al.,* 1996). This telomeric silencing in *Saccharomyces cerevisiae* involves linear spreading of repressive chromatin structures originating from the telomeric $C_{1-3}A$ repeats by means of the extended binding of heterochromatic proteins proximally from the distal end (Strahl-Bolsinger *et al.,* 1997). However, such telomeric silencing only reaches about 2–4 kb inward from these $C_{1-3}A$ repeats in wild-type cells. With respect to potentially analogous effects in human cells, human telomeres may not have the heterochromatic structure of yeast telomeres. For example, human telomeres were found to replicate throughout S phase, unlike yeast telomeres, which display the late replication associated with constitutive heterochromatin (Wright *et al.,* 1999). Although a telomere position effect was identified in human cells having a reporter gene adjacent to telomere repeats (Baur *et al.,* 2001), it was not observed in another study in which the test gene was >50 kb from the healed telomere (Ofir *et al.,* 1999). The 4q35 genes whose function in certain

skeletal muscles may be critically affected in *cis* by the presence of a short D4Z4 array are much more than 50 kb from the telomere, as described below. It is there-fore, unlikely that the proximity of the 4q35 D4Z4 array to the telomere has an effect on the heterochromatinization of the D4Z4 array or on heterochromatinization of 4q35 genes.

With respect to the loss-of-PEV model for FSHD, it has not been demonstrated that the repeat region of Chr4 in unaffected individuals is highly condensed, that this condensation spreads, or that FSHD patients have too little of this condensation. We have shown that unaffected individuals have one property indirectly associated with condensation of this DNA repeat, a high level of DNA methylation (Tsien *et al.*, 2001). However, many DNA regions are highly methylated without residing in constitutive heterochromatin (for example, see Magewu and Jones, 1994). Although there might be some FSHD-associated hypomethylation of D4Z4 repeats in short arrays, the D4Z4 repeats' extremely high CpG frequency (10% CpG) and general hypermethylation (Tsien *et al.*, 2001) would require massive demethylation in the short arrays to influence region-wide interactions.

We are currently studying histone modification, heterochromatin protein association, and chromatin nuclease sensitivity to evaluate the chromatin structure in pivotal regions at 4q35. In this chapter, I would like to consider, in the light of published findings, the PEV hypothesis and alternative hypotheses for how the decrease in copy number of D4Z4 repeats at 4q35 might result in FSHD-causing abnormal gene expression. I shall also summarize some of the challenges of elucidating the molecular aetiology of this syndrome.

17.2 Background and complicating factors in studies of the molecular genetic aetiology of FSHD

17.2.1 Large size of D4Z4 repeat array and associated FSHD candidate region and the importance of maintaining the natural spacing of FSHD-related DNA elements at 4q35

Among the factors that greatly complicate studies of how a short D4Z4 array at 4q35 causes FSHD is the large size of the normal D4Z4 arrays. In unaffected individuals, the D4Z4 arrays range from about 36–300 kb in length on each Chr4 homologue. These very large sizes make cloning normal D4Z4 arrays problematic because repeat-rich DNA clones are highly recombinogenic. Furthermore, the extremely high G+C content (73%) of the D4Z4 repeat probably makes the DNA sequence less stable upon cloning. Although the sizes of FSHD-associated D4Z4 arrays of 3.3–33 kb are much more manageable, the region of 4q35 that contains candidate FSHD genes consists of hundreds or thousands of kilobasepairs.

FSHD is a 4q35-associated disease in which not only the D4Z4 array but other sequences in *cis* are involved (see below). Since transcription control elements, such as silencers, can have profoundly different effects depending on their spatial relationship to the promoters they control (Ginjala *et al.*, 2002), it is important to maintain the natural spacings of regions in 4q35 that may be involved in FSHD. Given the uncertainties about which regions other than the D4Z4 array at 4q35 are involved in the disease, the paucity

of candidate genes close to the D4Z4 array on 4q35 and the strong homology between 10q26 and 4q35 for 42 kb proximally, it is

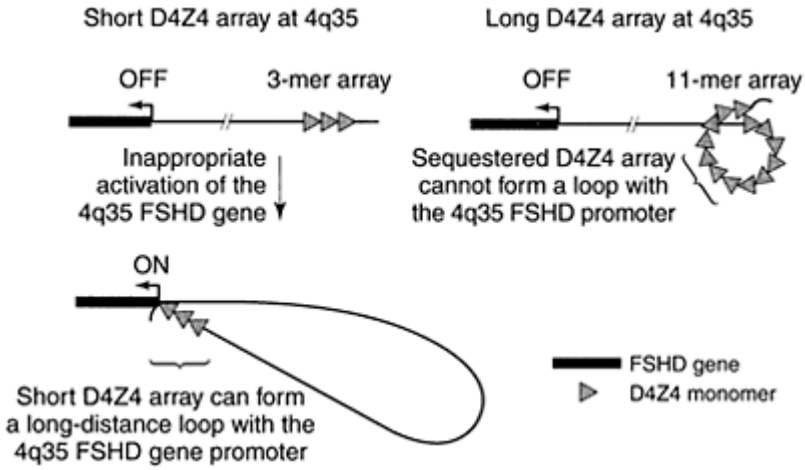

Figure 17.1 A Model for Short Array-Specific Gene Activation

A model for alternating looping at 4q35 controlling the FSHD phenotype. For simplicity, the short, FSHD-causing array (which can have 1–10 D424 repeats) is shown with only 3 repeats and the normal long array (which can have 11–100 repeats) with only 11 repeats. Also the 4q35 gene region that is initially impacted by a short array is displayed as a single gene although it might be a cluster of genes with one transcription-control element that interacts with a short D424 array in *cis*.

important to preserve a large region of 4q35 in studies of the effects of short D4Z4 arrays. Therefore, cloning only part of the D4Z4 repeat array is unlikely to simulate the effects of D4Z4 copy number on phenotype.

17.2.2 Homology between 4q35 and 10q26

There is extensive homology between 4q35 and 10q26 within their D4Z4 repeat arrays, proximally, and distally. Furthermore, both 4q and 10q D4Z4 arrays are similarly close to the telomeric $(TTAGGG)_n$ repeats and equally polymorphic in terms of copy number. Their homology presents many difficulties for analysis of the effects of partial deletion of the D4Z4 array at 4q35 but also offers clues as to models of how short arrays at 4q35 cause FSHD. The homology between the canonical D4Z4 repeat at 4q35 (GenBank AF117653, positions 5743–9038 and 9039–12 333) and that at 10q26 (GenBank NT_017795, positions 634, 423–637, 732, 637, 733–641, 042, 641, 043–644, 351, and 644, 352–647, 650) is 99%. The 42 kb region proximal to these repeat arrays between these two chromosomes is >95% homologous (van Geel *et al.*, 2002). In addition, the two polymorphic forms of the terminal portion of 4q35 proximal to the telomeric TTAGGG arrays, termed 4qA (from the beginning of the TTAGGG arrays to the D4Z4 arrays) and 4qB (~25 edkb; in the same position as 4qA and somewhat longer than 4qA) and the analogous region of 10q26 are highly homologous (van Geel *et al.*, 2002).

Among the small number of sequence differences between the typical 4q35 and 10q26 D4Z4 monomers, there are differences in a few restriction endonuclease cleavage sites (*Bln*I and *Xap*I; Lemmers *et al.*, 2001; van Geel *et al.*, 2002). The homology between 4q35 and 10q26 leads to rather frequent subtelomeric exchanges so that the canonical 10q repeats (the D4Z4 repeats with the *Bln*I sites) replace all or many of the 4q repeats (without *Bln*I sites) or *vice versa* in about 20–30% of the unaffected population (van Deutekom *et al.*, 1996a; Matsumura *et al.*, 2002). This homology and the tandem repetitive nature of D4Z4 arrays leads to intrachromosomal or interchromosomal (4q: 4q or 4q: 10q) exchanges or gene conversion and results in rather frequent somatic mosaicism (van der Maarel *et al.*, 2000). This is evidenced by 20–95% of the peripheral blood lymphocytes (PBL) in many *de novo* FSHD patients carrying the short 4q D4Z4 array (van der Maarel *et al.*, 2000). Also, up to 40% of the PBL in carriers may have the short D4Z4 array. Since somatic mosaicism for the short D4Z4 array at 4q can be found in unaffected carriers, sufficient normal skeletal muscle fibres may protect against the disease.

17.2.3 Homology between the D4Z4 subregions and various chromosomes

Sequences partially homologous to subregions of the D4Z4 monomer are found in various chromosomes but are especially common in the acrocentric chromosomes. With a D4Z4 probe for fluorescence *in situ* hybridization (FISH), annealing was seen at the short arms of all of the acrocentric chromosomes (Bayne *et al.*, 1994; Festenstein *et al.*, 1999; Ofir *et al.*, 1999; Baur *et al.*, 2001; McBurney *et al.*, 2002) as well as at 4q35, 10q26, 1qh (Iq12, juxtacentromeric heterochromatin, i.e. heterochromatin in 1q adjacent to the centromeric heterochromatin) (Hewitt *et al.*, 1994) and the centromeric region of Chr10 (Winokur *et al.*, 1994). One of the D4Z4 subregions that is repeated elsewhere in the genome is the Lsau homology sequence (89% homology from position −20 to +340, all positions within the D4Z4 region given relative to the *Kpn*I cleavage site; Hewitt *et al.*,

1994). It is present on the short arms of the acrocentric chromosomes, 1qh, and the pericentromeric regions of chromosomes 3 and 9, as well as Yq11 (Meneveri *et al.*, 1993). A sequence at position 1313 to 1780 shares homology with a low-copy repeat, *hhspm3* (Sp-0.3–8, 84% homology; Hewitt *et al.*, 1994) that is termed a sperm-specific hypomethylated sequence because it is hypomethylated in sperm but highly methylated in all postnatal somatic tissues (Zhang *et al.*, 1987), like the D4Z4 repeat (Tsien *et al.*, 2001).

Another subrepeat homology within the D4Z4 unit itself is a large open reading frame from 1792 to 2967, that could encode a 391 amino acid protein (DUX4). This is referred to as the double homeodomain region of DUX4 because of the two direct repeats encoding 60 amino acid sequences similar to the homeodomain motif found in a family of transcription factors that establish embryonic patterning in higher eukaryotes (Hewitt *et al.*, 1994). These two repeats are about 50% identical (Hewitt *et al.*, 1994; Lee *et al.*, 1995). Sequences homologous to this region are dispersed in the genome (Beckers *et al.*, 2001). A DNA probe from this region hybridizes to many bands in a Southern blot of total human DNA (Wright *et al.*, 1993) and >100 clones from a human phage DNA library hybridized to a probe from this region (Hewitt *et al.*, 1994). The *DUX4* 'gene' is immediately downstream of the *hhspm3*-like region which contains TACAA (a possible TATA box) and GGGGTGG (a possible Sp1 transcription factor-binding site). These sequences could provide promoter function for the *DUX4* open reading frame (ORF) (Gabriels *et al.*, 1999). However, no polyadenylation sequence is present downstream of this ORF. In a study in which cDNA libraries made from polyadenylated RNA were screened with this probe, several hybridizing clones were isolated but because of stop codons and frameshifts, they lacked an ORF that could produce an intact DUX4-like protein (Hewitt *et al.*, 1994). In another study, *DUX4*-like cDNA sequences were obtained from total RNA derived from a cancer cell line, which could be translated *in vitro* to give proteins similar, but not identical, to the putative DUX4 protein (Beckers *et al.*, 2001). By western blotting, cross-reacting proteins were found in the cell line (Ding *et al.*, 1998). The corresponding RNAs might be encoded by the acrocentric chromosomes (Hewitt *et al.*, 1994; Beckers *et al.*, 2001).

17.2.4 Homology between sequences proximal and distal to the D4Z4 array in 4q35/10q26 and various other chromosomal regions

In the 161 kb region of 4q35 proximal to the D4Z4 array, there is a very high percentage of repetitive sequence, especially, LINE1 elements which constitute 27% of total sequences in this region (van Geel *et al.*, 1999). This is a very gene-poor region, with *FRG1* and *FRG2* as the only identified genes (as opposed to the *TUBB4Q* pseudogene). Of these, only *FRG1* is specific to 4q35 because it is located 125 kb from the D4Z4 array far beyond the 42 kb region immediately proximal to the D4Z4 array that is shared between 4q35 and 10q26 (van Geel *et al.*, 1999, 2002, 1999). However, various parts of the 161 kb region centromeric to the 4q35 D4Z4 array exhibits homology to chromosomes other than Chr10. For example, using DNA from a rodent-human somatic cell hybrid panel, *FRG2* promoter-derived primer sequences (about 38 kb proximal to the D4Z4 array) were shown to amplify DNA from human chromosomes 3, 20 and 22 in addition to 4 and 10 (G.Jiang and M.Ehrlich, unpublished results). Another example of

the wide chromosomal distribution of sequences related to the D4Z4 array-proximal region is provided by a clone containing sequences surrounding and including a single D4Z4 repeat in opposite orientation from the D4Z4 array and 42 kb proximal to it. This clone hybridized not only with 4q35, 10q26, 1qh and a region near the rDNA on the acrocentric chromosomes, but also with regions on Chr20, ChrY, and the pericentromeric region of Chr9 (Altherr *et al.,* 1995). The *TUBB4Q* sequence (about 80 kb proximal to the 4q35 D4Z4 array), which appears to be unexpressed in various control samples and FSHD muscle despite having a long ORF, has homologous sequences on chromosomes 1, 9, 10, 12, 16, 18 and Y (van Geel *et al.,* 2000).

One of the two above-mentioned FSHD candidate genes, *FRG1,* was demonstrated by FISH to have homologous sequences at 9qh (9q juxtacentromeric heterochromatin), the short arms of all the acrocentric chromosomes, and the vicinity of the centromere in Chr20, in addition to 4q35 (van Deutekom *et al.,* 1996; Grewal *et al.,* 1999). Many of these are transcribed pseudogenes. By PCR amplification of a somatic cell hybrid panel, homologous sequences were found on the above chromosomes as well as on chromosomes 8 and 12 (van Deutekom *et al.,* 1996a). Also, sequences homologous to *ANTI* could be overamplified to give non-specific bands with exonic primers (reference for primers, Gabellini *et al.,* 2002) using as a template Chr20 DNA when the annealing temperature was 58°C (but not 60°C or higher; Jiang and Ehrlich, unpublished data).

Not only is there a very high degree of homology between 4q35 and 10q26 in the 42 kb proximal to their D4Z4 arrays, but also the region distal to the D4Z4 array on subtelomeric regions of 10q (Bengtsson *et al.,* 1994) closely resembles that of the two subtelomeric allelic regions 4qB and, especially, 4qA at the distal end of 4q35 (van Geel *et al.,* 2002). The homology between the 4qA allelic region and the distal end of 10q includes an ~8 kb array of tandem 68 bp repeats. Also, there is very high homology between this most distal region of 4q35, especially for the 4qB allelic region, and the most distal region of 4p (van Geel *et al.,* 2002). The region distal to the D4Z4 array on 4q35 is also homologous to the short arms of the acrocentric chromosomes and occasionally hybridized to the termini of other chromosomes as determined by FISH (Bengtsson *et al.,* 1994). With primers to the 4qA region (5′-GCTTAGGCAGATTTGGTG-3′ and 5′-TCCCAGATGTGTGAGAGT-3′), we could amplify by PCR all the human chromosomes in a monochromosomal somatic cell hybrid panel to obtain the expected size fragment (263 bp) as well as larger fragments (M.Ehrlich and G.Jiang, unpublished results). The presence of a high density of repeats in the subtelomeric region of 4q with homology to subtelomeric regions of other chromosomes is typical of subtelomeric regions (Flint *et al.,* 1997).

It is both extraordinary and fortunate that in this sea of interchromosomal homology in and around the D4Z4 array, there is one sequence extremely close to the array itself, which is almost unique in the genome; this is p13E-11, a 0.8 kb sequence only 111 bp from the beginning of the D4Z4 array on both 4q35 and 10q26. This sequence hybridizes in Southern blots with sequences from only one other chromosome, namely, the Y chromosome (Wright *et al.,* 1993; Hewitt *et al.,* 1994). The Y chromosome yields an invariant 9.5 kb hybridizing fragment in an EcoRI digest. This does not interfere with detection of the Chr4- and Chr10-derived D4Z4 arrays when the p13E-11 sequence is used as a probe to determine the size of the whole D4Z4 array in *Eco*RI/*Hin*dIII or

*Eco*RI/*Bln*I digests of genomic DNA subjected to pulsed-field gel electrophoresis (Wijmenga *et al.*, 1992; Deidda *et al.*, 1996; Upadhyaya and Cooper, 2002).

17.2.5 Tissue-specific and age-specific nature of the disease

FSHD is generally a muscle-specific disease initially affecting only a select group of skeletal muscles, mostly facial and shoulder girdle muscles, but often progressing to affect lower limb muscles (Lunt, 2000). The affected muscles that suffer atrophy are often asymmetrically located and only some muscle fibres in the given region of muscle are involved. Clinically significant extramuscular symptoms are infrequent in FSHD (Kissel, 1999). Extremely low copy numbers of 4q35 D4Z4 repeats are associated with the early onset of muscle symptoms, as early as in infancy (Tawil *et al.*, 1996; Lunt, 2000; Song *et al.*, 2000). However, the findings that symptoms are usually not manifest until the beginning of the second decade in life and that there is frequently an asymmetric distribution of affected fibres suggest a stochastic element in the molecular genetic pathway for expression of FSHD-associated muscle dysfunction.

17.3 Molecular clues about genetic aspects of FSHD

A short D4Z4 array itself does not cause FSHD because almost identical D4Z4 arrays on 10q26 that are equally short, do not result in a disease phenotype (Bakker *et al.*, 1995). As mentioned above, there are only a very small number of differences in the sequence of the typical 4q35 and 10q26 D4Z4 repeats, including within the putative promoter region of the double homeobox-type ORF. The candidate Sp1 and TATA sites (Gabriels *et al.*, 1999) identified in the 4q D4Z4 repeat (GenBank AF1 17653) are also present in the 10q D4Z4 repeat (GenBank, AL845259). Even a short canonical 4q35-type D4Z4 array on 10q26 does not cause the disease while a short 10q26-type array on 4q35 does (van Deutekom *et al.*, 1996). Therefore, it is the chromosomal position of the 4q35 D4Z4 array that links it to FSHD rather than the very infrequent sequence differences (1%) between the canonical 10q and 4q D4Z4 arrays. It can be inferred that FSHD results from the aberrant expression of some DNA sequences in *cis* to a short D4Z4 array at 4q35. Since 4q35 and 10q26 share extensive homology for 42 kb proximal to the D4Z4 array, the sequences which are differentially expressed in affected D4Z4 muscle relative to normal muscle, and are the immediate target of a short 4q35 D4Z4 array, must be at least 42 kb proximal to this array.

One putative gene region to be considered in constructing molecular genetic models for FSHD is *DUX4* within the D4Z4 array itself, which might encode a double homeobox protein (Hewitt *et al.*, 1994; Beckers *et al.*, 2001). In each copy of the 3.3 kb D4Z4 repeat, there is a putative promoter, the *DUX4* upstream region, mentioned above. However, it seems unlikely that differential expression from this promoter in patients and the general population is involved in FSHD. If transcription from this promoter were important in FSHD, then a decrease in D4Z4 repeat copy number to only 1–10 copies should somehow result in activation of expression from the putative *DUX4* promoter within the short array in certain FSHD skeletal muscle nuclei and yet, when as many as 100 copies of D4Z4 containing the same promoter are present in the 4q35 region in

unaffected individuals, such hypothetical deleterious expression would not occur. Therefore, in comparing 1-copy D4Z4 arrays at 4q in severely affected FSHD patients and 100-copy 4q D4Z4 arrays from unaffected individuals, a copy-number-dependent control of expression would have to activate expression from the putative *DUX4* promoter by more than two orders of magnitude owing to the presence of a short array. This seems implausible, and so I will consider only regions proximal to the D4Z4 array as sites of candidate FSHD genes.

The vast majority of individuals found to have <11 copies of the D4Z4 array at 4q35 manifest the disease with about 95% penetrance by age 20 (Lunt, 2000). Therefore, populations of asymptomatic adults are good controls for comparison to FSHD patients. Moreover, the importance of the D4Z4 copy number to the syndrome is seen in the finding that the disease is generally much worse (earlier onset and greater clinical severity) when the short 4q D4Z4 array is in the smaller size range for FSHD (Tawil *et al.*, 1996; Lunt, 2000; Song *et al.*, 2000). Nonetheless, factors other than the D4Z4 copy number at 4q35 modulate the clinical status. Males appear to be often more severely affected than females with the same size of the shortened array (Lunt, 2000). Although about 97% of FSHD patients have one allelic 4q35 D4Z4 array with 1–9 copies of the repeat, occasionally (3% or less of the general population) an individual with no symptoms of FSHD has <11 copies of the repeat at one Chr4 homologue (Upadhyaya *et al.*, 1997; Orrell *et al.*, 1999; van Overveld *et al.*, 2000). However, these unaffected individuals have only moderately reduced repeat copy numbers rather than very short arrays of five copies or fewer that are frequently seen in FSHD patients. With respect to individuals presenting with FSHD symptoms but not a shortened 4q D4Z4 array, it has been established that there is a rare non-Chr4-linked form of familiar FSHD of unknown aetiology (Tim *et al.*, 2001). In this chapter, by the term FSHD, I refer only to the disease involving a short D4Z4 array at 4q.

There is another polymorphic genetic element to be considered in the 4q35 region. In all of 80 examined FSHD patients, only one of the above-mentioned two D4Z4-distal polymorphisms was observed, 4qA (Lemmers *et al.*, 2002; van Geel *et al.*, 2002). By contrast, the unaffected population displays similar frequencies of the 4qA and 4qB alleles. The most notable difference between these allelic regions is the presence of an 8 kb region of tandem 68 bp Sau3A family repeats adjacent to the distal end of the D4Z4 array on the 4qA allele that is missing from the 4qB allele. However, the distal end of 10q is highly homologous to that of 4qA and includes the 8 kb array of tandem Sau3A repeats. Also noteworthy is the finding that the telomeric hexanucleotide repeats are closer to the D4Z4 arrays in 4qA than in 4qB (van Geel *et al.*, 2002).

Evidence that the 4qA allele does not merely predispose to shortening of the D4Z4 array comes from the analysis of DNA from six exceptional individuals with a short D4Z4 array but no FSHD symptoms (Lemmers *et al.*, 2002). Only three of these six healthy individuals who had a moderately contracted D4Z4 array (6–8 copies) displayed the 4qA allele and the others, the 4qB allele. It therefore appears that the 4qA allele is not simply necessary for generating short 4q D4Z4 arrays. However, from the very many control individuals screened for D4Z4 array sizes, there is no evidence that a short 4q35 D4Z4 array is unable to produce symptoms of FSHD in the presence of a 4qB allele in *cis* instead of a 4qA allele (R.Lemmers and S.van der Maarel, FSHD International Consortium Research Meeting, Oct, 2002). If the 4qA sequence has to work in concert

with an adjacent short D4Z4 array to cause FSHD, it is very surprising that numerous unaffected individuals with short, and even very short (1–5 copy number), D4Z4 arrays adjacent to a 4qB allele have not been identified. The most attractive explanation for the lack of short 4q35 D4Z4 arrays next to 4qB alleles is that embryonic lethality may result from these two genetic traits when they are present adjacent to one another.

Deletion mutations give some additional information that aids the evaluation of hypotheses for the molecular genetic aetiology of FSHD. A non-FSHD family was described in which a translocation was present between an acrocentric chromosome and the 4q35.2 region such that at least 250 kb of the distal end of 4q was replaced by chromatin from the p12 band of an acrocentric chromosome (Tupler *et al.*, 1996). It is important to note that this family retained 4q35 sequences beginning about 330 kb proximal to the telomeric end of 4q. The proband had hypogenitalism as his only symptom and his asymptomatic mother and maternal grandfather carried the same unbalanced translocation. From the lack of FSHD symptoms in this family, it was concluded that haploinsufficiency at 4q35 does not cause FSHD (Tupler *et al.*, 1996), although that conclusion pertains only to loss of the distal ~250–330 kb of 4q35. Therefore, FSHD could be due to a short 4q35 D4Z4 array causing haploinsufficiency for sequences more than ~300 kb proximal to the 4q telomere or to inappropriate activation of expression of 4q35 sequences.

A second spontaneous deletion in the 4q35 region also provides important information about the functionality of DNA sequence in the vicinity of the D4Z4 array on 4q. One patient with a sporadic case of FSHD had a deletion that included the 0.8 kb p13E-11 sequence immediately proximal to a short (2-copy) D4Z4 array at 4q35 (Lemmers *et al.*, 1998). Furthermore, the deletion extended proximally about 45 kb (S.van der Maarel, personal communication). Therefore, there cannot be an interaction between a shortened D4Z4 array on 4q and immediately proximal sequences that is necessary to establish the FSHD phenotype. Delimiting the extent of other proximal deletions will elucidate how much of this D4Z4 proximal sequence is not involved in FSHD. The patient who inherited the p13E-11 deletion had a *de novo* deletion of the D4Z4 array because his unaffected father and two brothers exhibited the p13E-11 deletion but had a high-copy-number of D4Z4 repeats in *cis* to that deletion. As the authors concluded, the proximal deletion apparently spread into the D4Z4 repeats in the patient (Lemmers *et al.*, 1998). This finding confirms the conclusion that no DNA sequences in the immediately proximal region to the 4q D4Z4 array have to be expressed on both normal Chr4 homologues or have to interact with normal-length D4Z4 arrays to prevent FSHD.

17.4 Difficulties with the PEV model for FSHD

Although the linear propagation of heterochromatinization model for PEV was attractive to apply to FSHD, it no longer seems to explain even *Drosophila* PEV (Wakimoto, 1998; Talbert and Henikoff, 2000). There is little evidence for linear spreading of gene silencing in *Drosophila* (Weiler and Wakimoto, 1995; Talbert and Henikoff, 2000). This spreading can be discontinuous, even at the cytological level (Belyaeva *et al.*, 1993). Furthermore, PEV-induced silencing in *Drosophila* can be facilitated by *trans* interactions between alleles, only one of which is in the vicinity of heterochromatin or a

multimerized DNA sequence (Dorer and Henikoff, 1997; Csink *et al.*, 2002). By definition, such *trans* interactions cannot occur by linear spreading. In addition, some examples of PEV in *Drosophila* involve great distances in *cis* over which heterochromatin would implausibly have to spread continuously, according to the linear propagation model.

In FSHD, PEV with linear propagation of heterochromatinization would involve heterochromatin spreading over long distances between candidate FSHD genes on 4q35 and the D4Z4 array. The two best candidate FSHD genes described in the literature are *FRG1* and *ANT1*, which are located 125 kb and ~4.8 Mb proximal to the D4Z4 array with no other identified FSHD candidate genes between them. They are both found on 4q, and not 10q, and so their postulated FSHD-specific dysregulation could explain why a short D4Z4 array at 4q can cause FSHD whereas one at 10q cannot. Of these two genes, *ANT1* is the much better candidate because it encodes an adenine nucleotide translocator that has been implicated in a myopathy (progressive external ophthalmoplegia) and predominantly expressed in heart and skeletal muscle (Gabellini *et al.*, 2002).

However, even more pertinent to considering any version of the PEV hypothesis for FSHD is the need for evidence that the D4Z4 arrays are themselves heterochromatic and a source of spreading of heterochromatinization. As mentioned above, there is no direct evidence for heterochromatinization of the large D4Z4 tandem arrays at 4q35 or 10q26. By chromatin immunoprecipitation (ChIP), we have investigated histone acetylation as an indicator of heterochromatic *vs.* euchromatic structure at subregions in 4q35. We could not examine the D4Z4 array itself because all six sets of PCR primers for subregions of D4Z4 that we tested were not specific for chromosomes 4 and 10 (G.Jiang, C.Sanchez, and M.Ehrlich, unpublished results). However, we could use primers for the p13E-11 region to assess histone acetylation immediately adjacent to (111 bp from) the D4Z4 array on chromosomes 4 and 10. We demonstrated that in peripheral blood mononuclear cell samples from six unaffected individuals, p13E-11chromatin exhibits a histone acetylation level typical of unexpressed euchromatin rather than that of constitutive heterochromatin (G.Jiang, F.Yang, and M.Ehrlich, unpublished results). That a sequence so close to the normal, long D4Z4 arrays does not exhibit the histone hypoacetylation of constitutive heterochromatin makes it highly unlikely that such arrays are sources of *cis*-spreading heterochromatinization. Although, it is not possible to do the ChIP analysis on muscle biopsies, we obtained the same results from lymphoblastoid cell lines and fibroblasts from many unaffected individuals. If there was copy number-dependent heterochromatinization, it would be expected to be found in all tissues, as is the case for pericentromeric heterochromatin. The tissue-specificity of FSHD is likely to derive from tissue-specific transcription factor(s) interacting with the proximal FSHD target gene(s) on 4q35.

One prediction of the PEV hypothesis can be tested on muscle biopsy samples. There were previous contradictory reports, based upon reverse-transcription PCR (RT-PCR) studies, as to whether *FRG1*, 125 kb proximal to the D4Z4 array at 4q35, is overexpressed in FSHD muscle and only weakly expressed in lymphocytes from unaffected individuals (van Deutekom *et al.*, 1996; Gabellini *et al.*, 2002), as would be predicted by the PEV model for FSHD. By end-point RT-PCR analysis, a very large extent of FSHD-specific overexpression of *FRG1* and *ANT1* was seen in muscle samples in a study by Gabellini *et al.* (2002). In the study by van Deutekom *et al.* (1996b), using

allele-specific polymorphisms and single-strand conformation polymorphism analysis of PCR-amplified muscle cDNA from four patients, no differences were seen in the RNA levels from the polymorphic *FRG1* alleles in each patient. In a control experiment, the authors found that mixtures of homozygous cDNAs allowed detection of a 30% reduction in transcript level.

The above two studies did not involve quantitative real-time PCR. Recent quantitative real-time RT-PCR and quantitative PCR-based histone acetylation chromatin immunoprecipitation analyses in our laboratory indicate that *FRG1* is expressed at moderate levels in lymphocytes from unaffected individuals (Jiang, G., Yang, F., and Ehrlich, M., unpublished data). Furthermore, no disease-associated increases were seen in *FRG1* levels in affected FSHD muscle biopsy samples from six individuals when compared to six analogous non-FSHD samples by real-time RT-PCR in which we used four different constitutively expressed RNAs as standards. These data are inconsistent with the PEV model for FSHD, especially because *FRG1* is the closest *bona fide* gene to the D4Z4 array. Given the lack of other identified genes (other than *FRG2* shared by 4q35 and 10q26) in the 161 kb proximal to the D4Z4 array on 4q35 and anywhere distal to the array, candidate 4q FSHD genes affected in *cis* by short D4Z4 arrays may be further distant than 161 kb from the array.

17.5 Other molecular genetic models for FSHD

17.5.1 General considerations

The conclusion that *cis* interactions between short D4Z4 arrays and distant 4q35 genes are involved in FSHD favours some molecular genetic models over others. Although it is possible that a short D4Z4 array induces repression and haploinsufficiency at a target gene more than 300 kb proximal to it, that seems much less likely than inappropriate activation of such a target gene. Since the target is so far away, it is more difficult to envisage long-lived repression associated with the short array than inefficient and possibly short-lived, yet deleterious, activation of expression of one or a few genes. Even transient gene activation in one nucleus of a muscle fibre might lead to a cascade of *trans* effects, for example, deregulated expression of transcriptional control or signalling proteins. Therefore, only activation models will be considered further.

A *caveat* in the analysis of candidate FSHD genes is that it is possible that a transcribed intergenic sequence, rather than a conventional protein-encoding gene, is the immediate downstream target of a shortened, disease-linked D4Z4 array. For example, in primary erythroid tissue at the murine β-globin *(Hbb)* locus, unexpected RNA synthesis was seen from an upstream transcription regulatory sequence (the LCR, see below) (Ashe *et al.*, 1997). Also, intergenic transcription was observed at this locus. Both types of non-gene transcription were independent of transcription of β-globin family genes. Transcripts that do not originate from protein-encoding genes or the classical structural RNA-encoding genes (rRNA, tRNA, 5S RNA) may influence gene expression in *cis* in various ways. For example, *XIST* RNA coats the whole inactive X chromosome in female mammals leading to repression throughout most of the chromosome (Brockdorff, 2002). In a different genetic system, there is evidence that a 100 kb *cis*-acting intergenic

transcription control region of the *Drosophila* bithorax complex plays a role in development that involves the formation of multiple non-coding transcripts from this region (Bae *et al.,* 2002). It has been proposed that this control of gene transcription by intergenic transcription in *cis* derives from the act of transcription in the long transcription-regulatory intergenic sequence rather than from accumulation of the corresponding intergenic transcripts (Drewell *et al.,* 2002). Another type of transcribed non-gene sequence to be considered is the newly discovered mammalian DNAs encoding micro RNAs (Lagos-Quintana *et al.,* 2003). These micro RNAs are 20–24 bp long and are processed from 70 bp or longer precursors. At least some of them control gene expression at the post-transcriptional level and so may be involved in human disease (Dostie *et al.,* 2003). However, it is estimated that there are not more than several hundred genes encoding microRNAs in the human genome (Lim *et al.,* 2003). The first gene(s) to be dysregulated in FSHD might be a cluster of co-regulated genes or atypical, non-protein-coding transcription units. In the subsequent discussion, for simplicity and because the number of known human genes greatly exceeds the number of DNA sequences currently associated with transcription control by non-gene transcripts, I will assume that the immediate target of short D4Z4 arrays is a *bona fide* gene at 4q35 and that this first target is a single gene. This as-yet-to-be-elucidated 4q35 gene whose abnormal expression in certain skeletal muscle nuclei in FSHD patients starts a chain of events leading to the characteristic symptoms of FSHD will be referred to as the FSHD gene.

Models for the molecular genetic basis of FSHD might involve localization of the FSHD gene at 4q35 allelic region to a repressive subnuclear compartment that depends upon whether a short D4Z4 array is present in *cis*. Localization to constitutive heterochromatin-rich subnuclear compartments has been observed and linked to repression of gene expression for some early lymphogenesis genes (Gasser, 2001). However, such a mechanism for the immediate-downstream deleterious effect of the short D4Z4 array at 4q35 is inconsistent with the above-mentioned recent data from our lab and the published findings of van Deutekom *et al.* showing no increase in affected FSHD muscle biopsies vs. control muscle biopsies in the expression of *FRG1* (van Deutekom *et al.,* 1996b). *FRG1* is the closest gene in the vicinity of the D4Z4 array that is not present at 10q26. Differences in subnuclear localization of 4q35 genes would be expected to have similar effects on gene expression from a large region. It is therefore unlikely that in unaffected individuals a 4q35 D4Z4 array that has more than ten copies of the repeat somehow causes repressive compartmentalization that does not occur when there are fewer than ten or 11 copies in affected muscle.

17.5.2 The long-distance looping hypothesis for transcriptional activation in FSHD

The evidence described in Section 17.3 indicates that two, and possibly three, types of 4q35 sequences cooperate in the initial interactions that respond to a short D4Z4 array at 4q35. These three kinds of sequences are the short, 4q35 D4Z4 arrays themselves; the FSHD gene, which is present on 4q35 proximal to and far from the D4Z4 array, and not on 10q26; and the distal 4qA allelic region. With respect to the 4qA region, as discussed above, there are only very small numbers of unaffected individuals who have been found

to have a short D4Z4 array adjacent to a 4qB allele, and these individuals never have a very short D4Z4 array, as do so many FSHD patients. To explain this finding and the lack of FSHD patients with a short D4Z4 array adjacent to a 4qB allele, the simplest hypothesis is that there is an embryonic-lethal effect of having the 4qB allele next to a short D4Z4 array. An interaction at 4q35 between a short D4Z4 array, 4qB, and the FSHD gene might lead to lethal expression of the FSHD gene in embryogenesis and not merely its inappropriate expression in a subpopulation of skeletal muscle nuclei, as is presumably the case in FSHD patients. The 8 kb tandem 68 bp repeat region unique to the 4qA allele might mitigate against this lethal interaction while permitting the FSHD-inducing intrachromosomal interactions. In that case, the 4qA region would not participate in FSHD-causing interactions, but rather would prevent intrachromosomal interactions from making the phenotype embryonic-lethal. In the following discussion, I will consider only interactions between the D4Z4 array and the FSHD gene.

Since, as explained above, available evidence argues against the loss of heterochromatin spreading from a short D4Z4 repeat to proximal 4q35 genes in FSHD, it is most likely that the communication between the short array and the FSHD gene, which is probably more than 161 kb away, occurs by looping. Such abnormal looping interactions might up-regulate transcription of a muscle-specific gene on 4q35 by direct chromatin–chromatin interactions. These interactions could facilitate delivery of positive transcription factors to the FSHD gene's promoter. Alternatively they could alter the structure of chromatin at the promoter of the muscle-specific gene, or influence the association of the gene region with the nuclear scaffold so as to locally affect transcription.

There is evidence for looping providing interactions between enhancers and promoters (Tolhuis *et al.*, 2002). However, even more pertinent to the FSHD-associated 4q35 subregion are recent studies demonstrating direct interactions between locus control regions (LCRs) and the rather distant genes that they positively control (Carter *et al.*, 2002; Tolhuis *et al.*, 2002). LCRs are operationally defined as *cis*-acting elements required for transgene expression that is approximately proportional to the number of integrated copies (Alami *et al.*, 2000). They can be much larger (up to 16 kb), than enhancers and can act over longer distances (50–70 kb) (Ashe *et al.*, 1997; Ho *et al.*, 2002). However, such interactions have not been described over hundreds of kb. Therefore, the very-long-distance looping that I am proposing for the D4Z4 array and FSHD gene is beyond the range of characterized LCRs. For example, indirect evidence from transgenic mice indicates that the murine β-globin LCR preferentially interacts with a sequence placed 7 kb downstream rather than the same sequence 43 kb downstream (Dillon *et al.*, 1997).

Long-distance looping that controls gene expression has also been postulated between *Drosophila* polycomb response elements (PREs) and their target genes and between PREs from different gene families separated by very large distances (Mahmoudi and Verrijzer, 2001). PREs are transcription-regulatory sequences important in early *Drosophila* differentiation that may act over long distances, up to 100 kb from the promoter, as in the bithorax complex (Mihaly *et al.*, 1998). PREs may cooperate with one another over yet longer distances. When PREs stimulate rather than repress gene expression, they are sometimes referred to as trithorax response elements (TREs). PREs can extend for hundreds of basepairs to several thousand basepairs. Complexes

containing polycomb group (PcG) or trithorax group (trxG) proteins can be recruited to and aggregate at PREs and may spread out in a linear fashion up to several kb from the PRE.

In human cells, evidence for transcription regulation over long distances has been described for the *PAX6* gene which has a transcription regulatory element more than 150 kb downstream of its P1 promoter and outside of the transcribed region of this gene (Kleinjan *et al.,* 2001). Upstream and downstream transcription control elements may cooperate with each other over this large region. Evidence suggests that disruption of the downstream regulatory sequence at one *PAX6* allelic region results in haploinsufficiency and anridia.

Evidence for very-long-distance chromatin interactions has been found in diverse eucaryotes. Loops between very distant portions of a *Drosophila* chromosome in association with PEV have been visualized cytologically (Dernburg *et al.,* 1996; Seum *et al.,* 2001). In *Arabidopsis* parenchymal cells, FISH analysis indicated the presence of 0.2–2 Mb euchromatic loops (Fransz *et al.,* 2002). In human cells, loops containing several megabases of DNA extending outwards from the surface of an interphase chromosome have been inferred from FISH analysis (Volpi *et al.,* 2000).

I propose that disease-associated long-distance loops involving the 3.3 kb D4Z4 repeat array and the FSHD gene can form only when a specific interaction between D4Z4 repeats 33 kb apart does not sequester the array. The intra-array interaction is proposed to occur in all normal-length (11–100-copy) D4Z4 arrays and to be dependent on topological overlapping of D4Z4 repeats 33 kb apart. These interactions between sufficiently far apart copies of D4Z4 within the array may depend on chromatin protein bridges between correctly spaced copies of the D4Z4 repeat and/or may be dependent on DNA homology. There are many precedents for homologous pairing of duplex DNA sequences in interphase in *Drosophila,* for example, silencing of the wild-type *brown* gene by an allele with a centric satellite DNA insertion (Henikoff, 2000). However, in mammalian cells, there is much less evidence for this pairing. The finding that more tandem copies of a transgene lead to more gene silencing in mammals and *Drosophila* (Sabl and Henikoff, 1996; Garrick *et al.,* 1998) might be explained by intra-array homology-dependent associations in interphase. The tendency of certain heterochromatic regions on different chromosomes to pair with homologous regions on other chromosomes in certain mammalian cell types (Haff and Schmid, 1991) suggests either pairing of homologous sequences in interphase or interactions between the same region-specific chromatin proteins in *trans*. The extraordinarily high G+C content of the D4Z4 repeat (73% G+C) as compared to the overall G+C content of human DNA (42%) may facilitate the hypothesized intra-array interactions between copies of the D4Z4 repeat. There could be constraints on the higher-order organization of chromatin in the D4Z4 array that could account for the threshold effect of D4Z4 copy number at 4q35 in FSHD. For example, a special type of stable loop might efficiently form only between portions of D4Z4 chromatin containing at least 11 tandem repeats.

In this long-distance looping model, short-distance interactions within the D4Z4 array preclude long-distance looping with an unidentified transcription regulatory region (FSHD transcription regulatory region) at 4q35 that governs expression of the yet-to-be-elucidated FSHD gene. According to this hypothesis, when the copy number is in the high-normal range at the D4Z4 array, there would be proportionately more short-distance

D4Z4 interactions than in the low-normal range. However, it is proposed that any amount of this intra-array quaternary structure interferes with inappropriate long-distance interactions with the FSHD transcription regulatory region, possibly by tethering the array so as to decrease its ability to engage in long-distance looping. It is difficult to speculate as to the nature of the structural 'ruler' (the biochemical device sensitive to the size of the array) that could distinguish 12 tandem copies of the 3.3 kb D4Z4 repeat (normal allelic region on 4q) from only nine copies (FSHD-causing allelic region on 4q). The nature of the higher-order structure of eucaryotic chromatin is still uncertain, for example, whether it involves a solenoidal or a zig-zag arrangement of the 30 nm chromatin fiber (Woodcock and Dimitrov, 2001). However, a higher-order structural limitation at the D4Z4 array that is sensitive to a threshold copy-number of the repeats seems the most probable parameter impacting upon gene expression at 4q35 so as to cause FSHD in response to a short D4Z4 array.

According to this model of long-distance looping for transcriptional activation in FSHD, it is predicted that D4Z4 arrays with >10 copies efftciently form the stable intra-array interactions, those with 5–10 copies of the D4Z4 repeat at 4q35 sometimes establish less stable intra-array interactions, and arrays with 1–4 copies almost never form these interactions. This would explain the above-mentioned increased severity of symptoms and decreased age at diagnosis associated with very short D4Z4 arrays at 4q35. The intramolecular nature of the chromatin interactions would explain the observed insensitivity of the short array-containing Chr4 to long D4Z4 arrays on the normal Chr4 homologue and on the Chr10 homologues. Activation of the FSHD transcription regulatory region by a single short D4Z4 array at 4q35 not sequestered by intra-array interactions would be consistent with the dominant nature of the disease. It has been suggested that unfacilitated, very long-distance chromatin interactions are so difficult to establish that they require very long cell cycle times or the lack of cycling to be generated at appreciable frequencies because they should be slow to form and mitosis should disrupt them (Dernburg *et al.*, 1996). This would be in accord with the development of symptoms usually only in the second decade in FSHD patients and would explain why a terminally differentiated cell type like muscle is particularly susceptible to inappropriate gene expression linked to short D4Z4 arrays at 4q35.

Interspersed repeat LINE-1 sequences in the 134-kb region (GenBank sequence AF146191) proximal to the D4Z4 array at 4q35 are about seven times more frequent than in the human genome as a whole (Gabriels *et al.*, 1999). Although most of them are highly truncated, they might play a role in enabling communication between a short D4Z4 array and the hypothesized, very distant FSHD transcription control region. This might involve the propagation of the proposed long-distance interaction by multiple shorter loops. The human X chromosome has an overall twofold enrichment in LINE-1 sequences, and the enrichment is most prominent in Xq13, which harbours the X-inactivation centre (Bailey *et al.*, 2000). It has been hypothesized that the density of LINE-1 sequences in the X chromosome serves to propagate the X-inactivation signal along the X chromosome that becomes inactivated. It has also been proposed for *Drosophila* that dispersed repeats help transmit long-distance chromatin interactions (Seum *et al.*, 2001). By computer searches (using Repeat Masker) in successive 50 kb regions of GenBank sequence AF250324 extending proximally from the 134 kb region next to the D4Z4 array, we found about a 3–6-fold over-representation of LINE-1 repeats

for another 200 kb. In the following 100 kb, the over-representation is lost but it is present again in the sub-sequent 100 kb. Therefore, LINE-1 sequences are positioned so that they might help to establish long-range interactions for at least 550 kb from the D4Z4 array at 4q35. In the region from 160–550 kb proximal to the D4Z4 array on 4q, there are a number of predicted genes, whose coding status remains to be determined. Although we have found by database analysis that there is also an over-representation of LINE-1 sequences in the 300 kb proximal to 10q's D4Z4 array, most hypotheses about FSHD, like the present one, assume that 10q lacks 4q's postulated muscle-specific gene(s) whose regulation is abnormally altered in response to a short D4Z4 array in *cis*.

In this long-distance looping hypothesis, interactions between the short D4Z4 array and the distant FSHD transcription control region are proposed to be positive while specific intra-array associations prevent these long-distance interactions. Sp1 has been inferred to bind the putative *DUX4* promoter from D4Z4 in transient expression assays using a reporter gene driven by this promoter-like region (Gabriels *et al.*, 1999). The promoter region with the wild-type Sp1 consensussequence binding site gave much higher expression levels than obtained from the same plasmid with a mutant Sp1 site. Several proteins have been shown by electrophoretic mobility supershift assays to bind specifically to a D4Z4 subsequence at or near the Sp1-like site (Gabellini *et al.*, 2002). These are YY1, which can function as a transcription activator or repressor; nucleolin, a major multifaceted nucleolar protein, which has DNA helicase, transcription activating/repressing, and RNA binding activities; and HMGB2, which has an architectural role in chromatin and helps to increase the flexibility of DNA so that DNA sequences can cooperate in various functional changes (e.g. transcriptional activation) in the genome (Srivastava and Pollard, 1999; Thomas and Seto, 1999; Grinstein *et al.*, 2002; Vallejo *et al.*, 2002). It has been suggested that HMGB2 facilitates associations between distant regions of the genome (Grinstein *et al.*, 2002). Moreover, nucleolin has been hypothesized to recruit functional multimolecular complexes at the base of chromatin loops of distant genes (Galande, 2002). This function is attributed to its ability to bind specifically to base-unpaired regions (BURs, typically 100–150 bp regions associated with matrix attachment regions with a propensity to underwind under negative superhelical strain) (Galande, 2002). These transcription regulatory sites can help to explain how long-distance chromatin-to-chromatin associations of a short D4Z4 array with the FSHD gene's transcription regulatory region at 4q35 lead to the postulated potentiation of inappropriate transcription of the FSHD gene.

17.6 Conclusions

Based upon available evidence and precedents from other genetic systems, I propose a new hypothesis for how a decrease in the number of copies of the 3.3 kb repeat in the subtelomeric region of Chr4 leads to abnormal gene regulation in *cis* that triggers FSHD. While the model is novel and only some aspects of it may be correct, FSHD appears to have a unique molecular aetiology and tests of the model may lead to our understanding new levels of transcriptional control. This model involves hypothetical and mutually incompatible higher-order chromatin structures, either specific short-distance interactions between correctly spaced repeats at the D4Z4 array, or very long-distance looping

between the array and an unidentified key transcription region located proximally. Efficient formation of the intra-array chromatin structure is proposed to require >10 copies of the repeat and to protect against inappropriate activation of the proximal FSHD-associated transcription control region present in *cis* at a considerable distance from the array. This long-distance looping hypothesis for transcriptional activation in FSHD is consistent with what is known about proteins that bind to the D4Z4 repeat, the nature of DNA sequences proximal and distal to the repeat array, and many features of FSHD. By contrast, the PEV hypothesis for FSHD is no longer an attractive model to explain this novel, intriguing, and very troubling disease.

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18.
Histological, immunocytochemical, molecular and ultrastructural characteristics of FSHD muscle

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

18.1 Introduction

The earliest histological descriptions of facioscapulohumeral muscular dystrophy (FSHD) precede its recognition as a separate clinical entity. Indeed it was the postmortem observations of Cruveilhiers in 1848 on a case of FSHD that were later interpreted by Duchenne in 1868, and were used by him to argue his case for the nosological separation of muscular dystrophy from the spinal muscular atrophies (Cruveilhiers, 1852; Duchenne, 1868). The terminology in this period can be confusing to the modern reader, so it is well to remember that *'d'atrophie progressive de l'enfance* refers to FSHD and that *'l'atrophie musculaire progressive, de l'adulte, type Aran-Duchenne'* is not Duchenne muscular dystrophy but spinal muscular atrophy (SMA). Landouzy and Déjérine in a well-argued paper (1884) described the postmortem features in a case of *d'atrophie progressive de l'enfance* in a young man who had died from tuberculosis. The postmortem clearly demonstrated primary muscle disease—*'au microscope…l'existence d'une atrophie simple des faisceaux primitifs'* whilst the nerves of the face, the affected muscles and *'les racines anterieures'* were *'absolument normaux'*. They emphasized the importance of distinguishing its myopathic nature from the neuropathic, *'myélopathique'*, nature of SMA. Since then, many authors have observed features that define a number of primary myopathies such as vacuolation and excess glycogen in the glycogenoses (Cardiff, 1966; Bartram *et al.*, 1995; Wolfe *et al.*, 2000), lipid accumulation in fatty acid oxidation defects (Angelini *et al.*, 1981; Turnbull *et al.*, 1987; Walton *et al.*, 1994); α-actinin containing rods in the nemaline myopathies (Yamaguchi *et al.*, 1982; Walton *et al.*, 1994; North *et al.*, 1997) or ragged-red fibres in mitochondrial myopathies (Stadhoulders and Sengers, 1987; Hammans *et al.*, 1993).

FSHD, however, has not shown any single disease-specific morphological abnormality. The features are mostly non-specific and include fibre necrosis and regeneration, increased variation in fibre size, increased numbers of internal nuclei, increased fibre-type variability, and connective tissue and fat proliferation (Brooke, 1986; Walton *et al.,* 1994). Some cases may show occasional, or marked, mononuclear cellular infiltrates, and features such as fibre type grouping and angulated fibres have previously been ascribed to 'neurogenic' causes, even leading some authors to adopt the term FSH 'neuromyopathy' (Une *et al.,* 1983). The origin of the small angular fibres, which are particularly apparent in less affected muscles, is uncertain as some features suggest they may be regenerating fibres (Dubowitz, 1985a; Brooke, 1986; Lin and Nonaka, 1991; Arahata *et al.,* 1995; Sewry and Dubowitz, 2001). It must be remembered, however, that many observations were made prior to the molecular era or the wide application of immunohistochemistry. Some caution in interpretation is therefore needed.

FSHD can be described, in the non-specific use of the term, as a limb girdle muscular dystrophy. In previous years, the limb girdle muscular dystrophies were a confusing group of disorders that evoked much debate and controversy. This changed with the cloning of the gene for Duchenne muscular dystrophy (Koenig *et al.,* 1987), the identification of the defective protein product, dystrophin (Hoffman *et al.,* 1987), and the localization of dystrophin to the sarcolemma (Arahata *et al.,* 1988). Immunocytochemistry has aided the discovery of a host of dystrophin-associated proteins leading to some clarification of the genetic cause of many of the limb girdle muscular dystrophies (Campbell, 1995; Bushby, 1999), as well as protein alterations secondary to the defective gene (Bönnemann *et al.,* 1995; Noguchi *et al.,* 1995; Duggan *et al.,* 1997; Taylor *et al.,* 1997; Herrmann *et al.,* 2000; Sandri *et al.,* 2001; Sewry and Dubowitz, 2001). Many of these proteins are structural proteins that form complexes associated with dystrophin: the dystroglycans, sarcoglycans and sarcospan, and the syntrophins, dystrobrevins with nNOS.

The dystrophin-associated complex (DGC) spans the plasma membrane, linking the cytoskeleton with the extracellular matrix (Bushby, 1999; Anderson *et al.,* 1999; Bönnemann and Finkel, 2002). Not all proteins defective in LGMDs, however, appear to be closely involved with the DGC. Caveolin-3 and dysferlin are membrane-bound proteins, independent of the DGC and dystrophin (Anderson *et al.,* 1999). Caveolin-3 appears to interact with phosphofructokinase, whilst calpain-3 is a calcium-dependent muscle-specific protease (van Ommen, 1995; Bushby, 1999). In spite of the huge advances in our understanding of these and many other muscular dystrophies, there has been great difficulty in identifying the underlying cause of FSHD, although genotype-phenotype correlations have been demonstrated.

At the time of initiation of this work, no systematic study combining histology, ultrastructure, immunocytochemistry and molecular aspects had been undertaken.

18.2 Patient characteristics

Eight unrelated patients with typical clinical histories of FSHD were included in the study, although molecular diagnostic confirmation had not been performed in four of the cases. All biopsies were taken from the quadriceps or deltoid muscle and in all of them

the biopsy site chosen was a muscle at grade 4 MRC power (Medical Research Council, 1976). There were six female and two male patients with an average age of 44 years 5 months and a range of 30–57 years. Mean age of onset was 18.6 years, although the range of onset was from 11 to 36 years. In all patients, but one, symptoms were noted at 20 years or under. Mean duration of symptoms was 25.8 years, with a range of 9–39 years. Apart from the skewed sex distribution, this would represent a typical distribution of classic adult-onset FSHD cases.

18.3 Biopsy site

As with other neuromuscular diseases, taking a muscle biopsy in suspected FSHD can help establish the clinical diagnosis. Where there is a clear clinical indication, for example typical clinical features or known family history, molecular diagnosis should be the first line of diagnosis. Even where the diagnosis is less certain, since there are no pathognomonic histopathological features, molecular diagnosis should remain the first line of investigation. In sporadic and atypical cases or in those cases with a normal molecular test result, the muscle biopsy remains important. There has been much discussion about which muscle is most suitable for biopsy. It is critical that the selected muscle reflects the clinical disease, but muscles that are too diseased and weak (i.e. MRC grade 4 or less) are likely to show little more than severe end-stage fibrosis and fatty infiltration (see *Figure 18.1b*). Thus the subscapularis and supraspinatus are likely to be too diseased (and too poorly studied) to be suitable candidate sites for muscle biopsy. Dubowitz and others favour the deltoid or vastus lateralis because of the much greater experience and knowledge of pathological changes in these muscles, whereas Bodensteiner and Schochet favour the supraspinatus (Dubowitz, 1985a; Bodensteiner and Schochet, 1986). These sites are particularly suitable early in the course of the disease, but in older cases the deltoid, and often the vastus lateralis, may be severely affected, in which case a less damaged and more distal muscle should be chosen, such as the biceps brachii (Dubowitz, 1985a; Lin and Nonaka, 1991; Arahata *et al.*, 1995).

Careful clinical selection is therefore essential, but even where great care has been taken, it is our experience that some biopsies will be more severely or mildly affected than anticipated on clinical grounds alone. With the better accessibility of imaging techniques such as muscle MRI or ultrasonography, suitable muscles can be identified and so avoid the need for a repeated biopsy (Dubowitz, 1985b; Chan and Liu, 2002; Mercuri, 2002a, 2002b). Once a biopsy site has been selected, it is important to avoid the focally destructive effects of EMG needles and hypodermic needles which can result in artefactual myopathic appearances in the needle tract, if these areas are biopsied. The practical implication is that a muscle biopsy should not be taken from a previously needled site, and conversely EMG studies or intramuscular injection should be avoided at sites chosen for muscle biopsy (Engel, 1967).

18.4 Sample handling *(Table 18.1)*

Samples collected at the theatre were divided into four parts. Samples to be used for immunocytochemistry (ICC), calpain-3 studies, and molecular analysis, were frozen to −70°C within 15 minutes of sampling. Samples for ICC were orientated on a cork tile in transverse section with the muscle facing upwards. They were then frozen in isopentane chilled to its freeze–thaw temperature in a liquid nitrogen

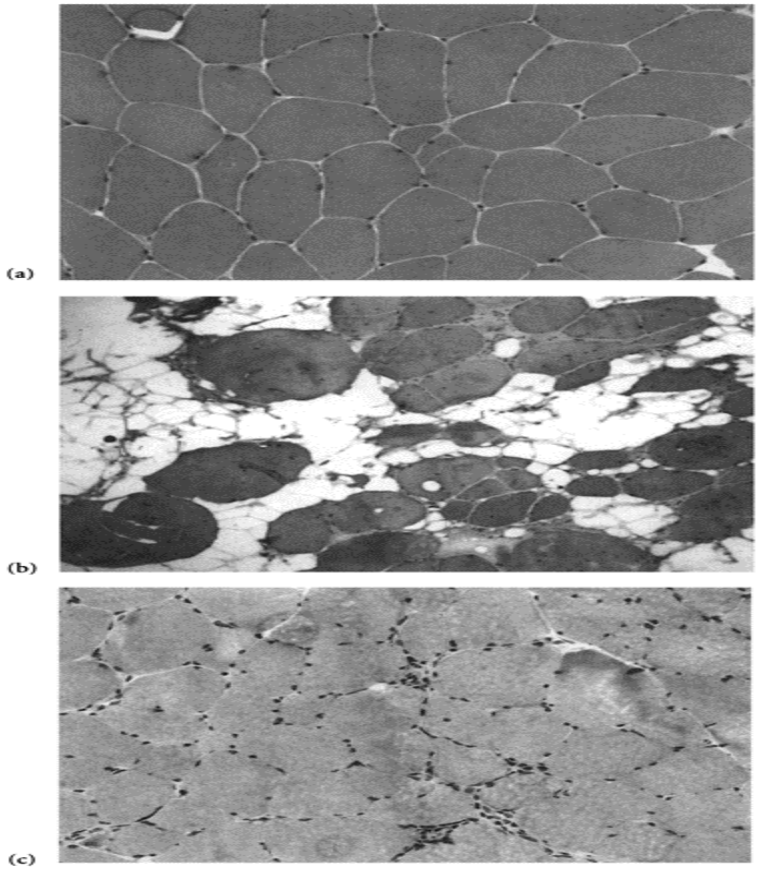

Figure 18.1 Muscle biopsies with varying degrees of pathology from three cases of facioscapulohumeral dystrophy. Note in (a) only mild changes with occasional atrophic fibres, (b) extensive fibrosis and fatty

tissue replacement, and (c) a focal area of cellularity in addition to fibre size variation.

bath. Freezing was continued for at least 1 minute following which samples were transferred to a prechilled universal container (containing snow-ice) kept ready and waiting in the liquid nitrogen bath. In order to ensure that no fluctuations in temperature occurred during transfer, samples were transferred to a −70°C freezer, still floating in liquid nitrogen (within the universal container).

Samples were stored at −70°C and then transported to Dr. Caroline Sewry.

Table 18.1 Details of proteins studied

Protein studied	Antibody used
Dystrophin	dys-1, dys-2 (c-terminal), dys-3, Dy4/6D3 (rod), Dy8/6C5 (C term) Novocastra NCL
Utrophin	Mancho 7 (gift of Prof G Morris)
α-sarcoglycan	Novocastra NCL-α-Sarc
β-sarcoglycan	Clone βSarc/5B1 Novocastra NCL-β-Sarc
γ-sarcoglycan	Clone 35DAG/21B5 Novocastra-γ-Sarc
δ-sarcoglycan	Clone δSarc3/12C1 Novocastra-δ-Sarc
α-dystroglycan	Clone V1A4 Upstate biotechnology
β-dystroglycan	Clone 43DAG1/8D5 Novocastra-β-DG
Dysferlin	NCLHamlet Novocastra Clone Ham1/7B6
Emerin	Clone 8A1 (gift of Prof G Morris, host institution)
Caveolin	Clone 26 Transduction laboratories C38320
HLA1	W632 Novocastra NCL-HLA-ABC
Fetal myosin	MHCn Novocastra
Slow myosin	MHCs Novocastra
Fast myosin	MHCf Novocastra
Spectrin	Novocastra NCL-SPEC
nNOS	Santa cruzNOS-1 sc648 (rabbit polyclonal)
α2-laminin (merosin)	Chemicon MAB1922
α5-laminin	Chemicon 1924
β1-laminin	Chemicon 1921
γ1-laminin	Chemicon1920

18.5 Immunoblotting (western blot) studies

Immunoblotting was performed by Dr Louise Anderson, University Medical School, Newcastle-upon-Tyne, UK. The profile of bands for α2-laminin, calpain-3 and dysferlin were all examined using immunoblots. *Table 18.2* provides details on the antibodies used.

Table 18.2 Immunoblot proteins studied

Protein	Antibody
Calpain-3	Calp3d/2C4 (N terminus), Calp3c/12A2
α2-laminin (merosin)	Commercial α2 (80 kDa fragment)
Dysferlin	NCL-hamlet (exon 53)

18.6 Ultrastructure

Samples allocated to the ultrastructural study were fixed for 3–4 hours in freshly prepared 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 at room temperature and post-fixed for 2 hours in 2% osmium tetroxide in veronal acetate buffer pH 7.3. The tissues were dehydrated through a graded series of ethanols, cleared in propylene oxide and embedded in Araldite epoxy resin. The embedded muscles were sectioned using a Riechert Ultracut E. 1 μm semithin sections were routinely stained with toluidine blue to view under a light microscope, whilst the ultrathin sections were stained with uranyl acetate and lead citrate, and viewed and photographed using a Jeol EX 1200 electron microscope.

18.7 Molecular analysis of FSHD

DNA was isolated from lymphocytes, fibroblasts and muscle biopsies (when available) using a standard protocol (Upadhyaya *et al.,* 1997). Five to ten μg DNA were digested with *Eco*RI, *Eco*RI/*Bln*I and fractionated on 0.5% agarose gel for 48 h at 0.5 v/cm and Southern blotted onto Hybond N (Amersham). DNA on the membrane was hybridized with [^{32}P] labelled DNA probe p13E-11 and autoradiographs were visualized. The smallest *Eco*RI/*Bln*I fragment associated with the FSHD disease allele was scored (Upadhyaya and Cooper, 2002). DNA could not be extracted from three muscle biopsies owing to the excessive fat content and from another two insufficient DNA was available for Southern blot analysis. *Eco*RI/*Bln*I fragments smaller than 35 kb were observed in lymphocyte DNA from seven patients. In another patient the *Eco*RI/*Bln*I fragment size was 48 kb. We observed similarsized fragments in five fibroblast- and two muscle-derived DNA samples, illustrating that similarly sized small fragments are present in different tissue types from the same FSHD patient. RNA has been extracted from some of the muscle biopsies for gene expression analysis.

18.8 Light microscopy

As a muscular dystrophy, one might expect most, if not all, FSHD biopsies to show prominent dystrophic features, with fibre necrosis and fibrosis. However in many cases disease progression is slow and muscle involvement variable, so that even where there is significant clinical disease many biopsies show only minor changes, and routine H&E sections may appear surprisingly normal (Dubowitz, 1985a; Bodensteiner and Schochet, 1986; Lin and Nonaka, 1991). On the other hand, muscle involvement may be so selective that a biopsy of a severely affected muscle is only able to show severe end-stage features such as extensive fibrosis and replacement with fat, helping to confirm the dystrophic process, but shedding no light as to the type of dystrophy (Bodensteiner and Schochet, 1986; Brooke, 1986; Schmalbruch, 1992; *Figure 18.1b*). Between these extremes, however, most biopsies will show abnormal variation in fibre size, an increase in the number of internal nuclei, and often little else (Munsat *et al.*, 1972; Dubowitz, 1985a; Lin and Nonaka, 1991; Schmalbruch, 1992; Sewry and Dubowitz, 2001). Fibre size analysis shows an increase in both hypertrophic and atrophic fibres (Brooke and Engel, 1967; Dubowitz, 1985a; Sewry and Dubowitz, 2001). Although small angular fibres may be prominent, mean fibre size is usually increased (Dubowitz, 1985a). These changes, as in other dystrophies, affect both type I and type II fibres (Brooke and Engel, 1967). There is rarely any fibre type disproportion or predominance, but the presence of many small type I fibres can give an impression of type II predominance. If fibre predominance is apparent it is likely to be of type II fibres, and fibre deficiency of type I (Dubowitz, 1985a; Lin and Nonaka, 1991). Oxidative enzymes may show unevenness of stain, moth-eaten fibres, or whorled fibres (Dubowitz, 1985a; Schmalbruch, 1992). Lobulated fibres with prominent peripheral mitochondrial clusters have also been reported in FSHD (Bethlem *et al.*, 1973; Lin and Nonaka, 1991; Nakagawa *et al.*, 1996, 1997; Saito *et al.*, 2000), but such fibres are rarely a feature of biopsies from children (Guerard *et al.*, 1985), although they have been described (Nakagawa *et al.*, 1996). Rarely, these have been sufficiently prominent to invoke the term Ragged Red Fibre, but no mitochondrial abnormality has been identified (Nakagawa *et al.*, 1996). Two features that are somewhat more characteristic, but by no means pathognomonic include (i) inflammation and (ii) the presence of small angular fibres.

Small angular fibres (SAF)

Small angular fibres, often barely apparent because they are squeezed tightly between fibres, are a particularly common, but not universal, feature of FSHD (Bethlem *et al.*, 1973; Brooke, 1986; Lin and Nonaka, 1991; Sewry and Dubowitz, 2001). They can also occur in neurogenic disorders including spinal muscular atrophy and motor neuron disease (Lin and Nonaka, 1991; Bossen, 2000), suggesting to some that they indicate denervation, particularly when seen together with other ‘neurogenic’ features such as fibre grouping or a ‘neurogenic’ EMG. This has helped to fuel the debate about the myogenic or neurogenic primary aetiology of FSHD (Bethlem *et al.*, 1973; Furukawa, 1995). They are also a prominent feature in the more recently delineated myotonic dystrophy type 2, DM2 (also known as proximal myotonic myopathy, PROMM)—another autosomal dominant dystrophy sometimes with significant phenotypic overlap

with FSHD (Vihola *et al.*, 2003). However, in FSHD type 1 fibre atrophy predominates, the small fibres tending to be of type 1; by contrast, in DM2 there is preferential type 2 fibre atrophy, frequent central nuclei (as in DM1), and large numbers of very small fibres (diameters <20 μm) with nuclear clumps (Vihola *et.al.*, 2003).

Regeneration, a key feature of muscular dystrophy, has long been described in FSHD biopsies (Munsat *et al.*, 1972; Sartore *et al.*, 1982; Tupler *et al.*, 1998). Lin and Nonaka (1991) compared biopsy features of the small angular fibres seen in FSHD and those of patients with spinal muscular atrophy (SMA) and one patient with amyotrophic lateral sclerosis and concluded that the small angular fibres of FSHD were the product of regeneration, rather than of denervation. The demonstration that the small angular fibres express fetal myosin and MHC class I antigens would support this conclusion (Tupler *et al.*, 1998; Sewry and Dubowitz, 2001; *Figure 18.2b*). Similarly, the small groups of atrophic fibres, previously thought to suggest denervation, express fetal myosin (Dubowitz, 1985b; Tupler *et al.*, 1998; Sewry and Dubowitz, 2001). Arahata *et al.* (1995) also described chronic dystrophic changes and regenerative fibres in all FSHD biopsies examined. However, the main thrust of their paper was not the small angular fibres but the inflammatory cells frequently encountered in FSHD biopsies.

Inflammatory infiltrates

Inflammatory infiltrates are a well-recognized and frequent finding in FSHD (Wulf *et al.*, 1982; Arahata et al., 1995; Nakagawa et al., 1997; Fitzsimons, 1999; Sewry and Dubowitz, 2001; and *Figure 18.1*). Depending on classification, they have been described in 21–100% of cases (Dubowitz, 1985a; Bodensteiner and Schochet, 1986; Arahata *et al.*, 1995; Nakagawa *et al.*, 1997) and although characteristic, they are not a pathognomonic feature of FSHD biopsies. Inflammation can be sufficiently marked to raise suspicion of polymyositis, so that many patients have been initially treated for this, leading some authors to advocate at least a therapeutic trial of steroids (Munsat *et al.*, 1972; McGarry *et al.*, 1983; Bacq *et al.*, 1985; Brooke, 1986).

Both muscle pain, a particularly troublesome feature of FSHD (Hughes, 1971; Padberg, 1982; Bacq *et al.*, 1985; Bushby *et al.*, 1998) and, rarely, a very high creatinine kinase (CK) can occur early. Muscle tenderness, however, is unusual and the inflammatory response and CK levels do not correlate with symptoms—

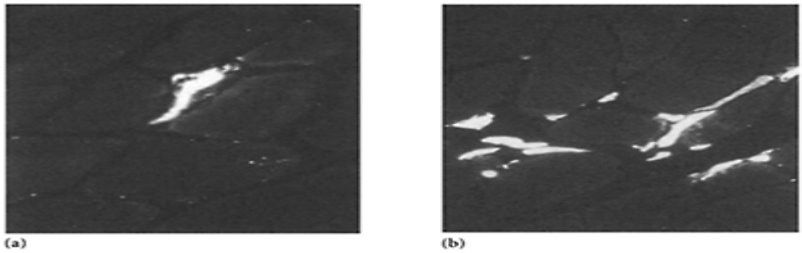

Figure 18.2 Muscle biopsies immimolabelled with an antibody to fetal myosis showing (a) a single

angulated fibre, and (b) a cluster of small positive fibres.

both marked inflammatory response being seen in painfree subjects, and no inflammation in patients with marked myalgia and very high CK. Bacq *et al.* (1985) did not find any evidence for raised biological markers of inflammation and on both light and electron microscopy were unable to demonstrate capillary abnormalities to support the case for a hereditary polymyositis with an FSH distribution. Arahata *et al.* examined the mononuclear cells from 18 Japanese FSHD subjects, all with a shortened *Eco*RI fragment (<28 kb). A marked inflammatory infiltrate was seen in 33% of biopsies. Although blood vessels were frequently surrounded by mononuclear cells (often $T4^+$ and $T8^+$ T-cells), these were presumed to be passing through the vessel wall (Arahata *et al.,* 1995). There was no invasion of non-necrotic fibres with $T8^+$, $performin^+$, nor granzyme A^+ cells, suggesting that a cytotoxic T-cell mediated immune reaction was not the primary cause of muscle damage in FSHD, and therefore that the inflammatory response was different from that seen in the inflammatory myopathies, polymyositis, dermatomyositis or inclusion body myositis. This would concur with many other reports and our own experience where for the most part only degenerating fibres were invaded (Lin and Nonaka, 1991).

Inflammation is neither a consistent nor a specific feature of FSHD biopsies, and may be seen in many other dystrophies, or myopathies, including Duchenne muscular dystrophy (Brooke and Engel, 1967; Brooke, 1986; Dubowitz, 1985a; McNally *et al.*, 2000). A prominent inflammatory response is frequently seen in dysferlinopathies, where it may be very marked and has also led to diagnostic confusion with polymyositis (McNally *et al.*, 2000; Gallardo *et al.*, 2001; Confalonieri *et al.*, 2003). Just like FSHD, there has been no clinical response to a therapeutic trial of steroids in the dysferlinopathies (McNally *et al.*, 2000). Gallardo *et al.* describe the inflammation, together with perivascular infiltrates, in 69% of their dysferlinopathy patients (Gallardo *et al.*, 2001). Like the FSHD biopsies examined by Arahata *et al.* only degenerating fibres appeared to be invaded, suggesting that this too is an inflammatory response to degenerating fibres, rather than a primary cause of the necrosis (Arahata *et al.*, 1995; McNally *et al.*, 2000; Gallardo *et al.*, 2001; Confalonieiri, P., *et al.*, 2003).

18.9 Immunocytochemistry

In recent years, immunocytochemistry has revolutionized our understanding of defective muscle proteins and their importance in muscle disease. Dystrophin, the defective protein in DMD and BMD, was the first to be identified (Hoffman *et al.*, 1987; Arahata *et al.*, 1988), and has since proved to be linked with an increasing number of proteins, both internal and external to the plasma membrane. Abnormalities in each sarcoglycan are now known to be the primary cause of four autosomal recessive limb girdle muscular dystrophies, and mutations in laminin-α2 (also known as merosin) to be the cause of one form of congenital muscular dystrophy (Campbell, 1995; Bushby, 1999). All these diseases present with skeletal muscle weakness, usually of a limb girdle distribution. It is also now known that abnormalities in one protein may lead to secondary changes in

others, such as up-regulation (e.g. utrophin in DMD: Helliwell *et al.*, 1992; Taylor *et al.*, 1997; and utrophin in FSHD: Tupler *et al.*, 1998), or down-regulation/loss, (e.g. the sarcoglycans in the sarcoglycanopathies or nNOS in DMD (Bönnemann *et al.*, 1995; Anderson *et al.*, 1999; Sewry and Dubowitz, 2001; Crosbie *et al.*, 2002).

Several of these secondary changes are of diagnostic value in assessing muscle biopsies. In addition, whilst earlier studies showed genetic heterogeneity but relative phenotypic homogeneity, more recent discoveries have shown increasing phenotypic heterogeneity and a broadening of the clinical spectra. Thus, abnormalities in the same protein can be associated with different or variable clinical phenotypes, i.e. different clinical diseases have now been shown to be due to allelic alterations in the same gene, and even in some cases the same mutation, for example dysferlin in Miyoshi myopathy and LGMD2B (Illarioshkin *et al.*, 2000; Ueyama *et al.*, 2001). FSHD also presents with a limb girdle distribution and many biopsies from cases presenting without a prior family history will have been studied with antibodies to the newly identified proteins associated with the limb girdle dystrophies. No consistent abnormality has become apparent despite the wide range of proteins studied (Tupler *et al.*, 1998). In some instances, FSHD biopsies have been used as controls for other studies with nothing abnormal being noted (Arahata *et al.*, 1988), whilst other authors have focused on particular proteins of interest (see below). The eight FSHD biopsies studied by the authors (from seven molecularly proven cases), were also examined using antibodies against a wide range of proteins *(Table 18.1)* (Rogers *et al.*, 2001). No consistent abnormality was noted, although occasional non-specific changes could be seen in some biopsies. MHC class I staining was increased in one sample but was normal in the other biopsies. In two samples, there was a slight reduction in α2-laminin expression. Expression of all other antibodies including dystrophin, utrophin, the sarcoglycans, dystroglycans, emerin, spectrin, the myosins, nNOS, and dysferlin was within normal limits. Similarly immunoblot studies of calpain-3 were also normal (Rogers *et al.*, 2001). The increase in MHC class I expression seen in one case adds to the debate about postinfective induction of disease.

Tupler *et al.* (1998) described twins who were clinically discordant for FSHD. Muscle biopsy of the more severely affected twin showed increased expression of MHC class I in both mature as well as immature fibres expressing fetal myosin. The more severely affected twin had had a rabies vaccination, which led the authors to speculate as to whether the severity of this condition might have had an immunological dimension. The presence of inflammation in many cases would lend some support to this hypothesis. Against this is the fact that, although prominent in some cases, inflammation is not a feature of all biopsies. As described above, it is also seen to a greater or lesser extent in many other dystrophies with large inflammatory infiltrates, at levels similar to those seen in FSHD, being described in a half of biopsies from patients with LGMD2B, a dysferlinopathy (Anderson *et al.*, 1999; McNally *et al.*, 2000; Gallardo *et al.*, 2001; Confalonieri *et al.*, 2003).

Other authors have used immunohistochemistry to focus on particular proteins of interest—such as the inflammatory response (Arahata *et al.*, 1995; see above) or the fibroblast growth factors (Saito *et al.*, 2000; Mongini *et al.*, 2002). Marked overexpression of FGF1 and FGF2, and FGF-R4 (an FGF receptor) was reported by Saito *et al.* (2002) in a severe case of molecularly proven FSHD. However, most of the remaining eight cases of FSHD expressed both FGF1 and FGF2 at similar levels to those

seen in Becker muscular dystrophy or limb girdle muscular dystrophy. Preliminary results of a similar study of mildly affected patients with FSHD did not reveal altered expression of FGF1, FGF2 nor FGF-R4 (Mongini *et al.,* 2002). Moderate expression of these factors was seen in Duchenne muscular dystrophy. Since fibroblast growth factors are involved in the proliferation of fibroblasts, the increased expression in DMD and the severe case of FSHD is more likely to reflect the level of abnormal muscle fibrosis rather than to be the cause of FSHD (Mongini *et al.,* 2002).

Caspase-3 activation is one of the more terminal events that leads to myofibre apoptosis. This follows the cascade activation of other caspases and proteases leading to a likely common pathway involving caspase-3. These have been examined in both FSHD and DMD human muscle biopsies (Sandri *et al.,* 2001). As with the fibroblast growth factors, the exact profile of changes in the two diseases is not the same, different caspases being activated higher up the chain, which may reflect disease-specificity, age-relatedness, or clinical severity, although these were not demonstrated by Sandri *et al.* Correlation of caspase-3 and bax expression led Sandri *et al.* to propose a role for mitochondria in this myofibre cell death pathway, and to suggest a role for caspase inhibitors in the management of muscular dystrophies.

18.10 Electron microscopy

There is a paucity of information about the ultrastructural changes seen in FSHD muscle biopsies. As with light microscopy and immunohistochemistry, no specific ultrastructural feature has been consistently demonstrated (Munsat *et al.,* 1972; Bacq *et al.,* 1985). In reports where EM features have been described, the emphasis has often focused on specific aspects such as the lobulated fibres described by Bethlem *et al.* or the capillary changes associated with polymyositis (Munsat *et al.,* 1972; Bethlem *et al.,* 1973; Bacq *et al.,* 1985). In our studies of eight biopsies, we undertook a systematic ultrastructural examination of the myofibrillar structure, sarcolemma and organelles. In some cases, even those exhibiting marked changes on light microscopy, EM features were remarkably inconspicuous.

18.10.1 Myofibrillar structure

Myofibrillar structure of individual fibres was well preserved, even including cases with extensive fibrofatty change *(Figure 18.3).* Increased space between myofibrils was observed in some fibres but is a non-specific feature of many neuromuscular conditions (Munsat *et al.,* 1972; Brooke, 1986; Walton *et al.,* 1994). Similarly, occasional splitting and branching of myofibrillar bundles was seen and may occur in normal biopsies. However, the level seen in some of the authors' cases was greater than would be expected *(Figure 18.3).* It should be noted, however, that *myofibrillar* splitting should not be confused with the *fibre* splitting seen under light microscopy, especially as it has been reported that lack of fibre splitting can be useful in distinguishing FSHD from other myopathies (Dubowitz, 1985a)

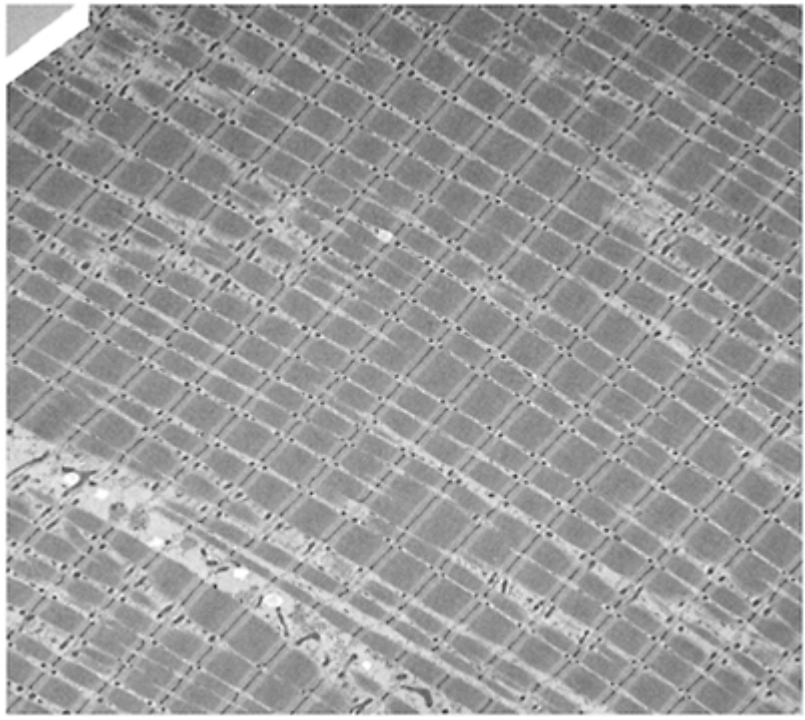

Figure 18.3 Low power view from a severe case of FSHD showing splitting and branching of myofibrillar bundles, but preservation of the myofibrillar structure of individual fibres.

18.10.2 Mitochondria

Mitochondrial numbers and localization vary a lot between the three major fibre types, and it is difficult to distinguish fibre types at the ultrastructural level in human muscle. Type IIb (fast-twitch glycolytic) fibres tend to have fewer and smaller mitochondria in a transverse orientation. Type IIa (non-glycolytic fasttwitch) fibres tend to have larger longitudinally orientated mitochondria. And type I (slow-twitch) fibres tend to have the greatest number of variably sized mitochondria arranged in pairs at the A/I junction of each sarcomere (Stadhouders and Sengers, 1987). Some degree of subsarcolemmal clustering is also a normal feature of type I fibres (Cullen and Landon, 2001). Bearing all these facts in mind, some degree of mitochondrial variation is inevitable, including a diminution in numbers or an uneven distribution of mitochondria. Occasional large, bizarre-shaped mitochondria fill some of the larger spaces. This may reflect true myofibrillar loss and stimulation of mitochondrial branching and duplication to fill the

'dead space' *(Figure 18.4)*. Subsarcolemmal clumping of mitochondria was seen in two of the authors' cases *(Figure 18.5)* and is a feature of lobulated fibres (Bethlem *et al.*,

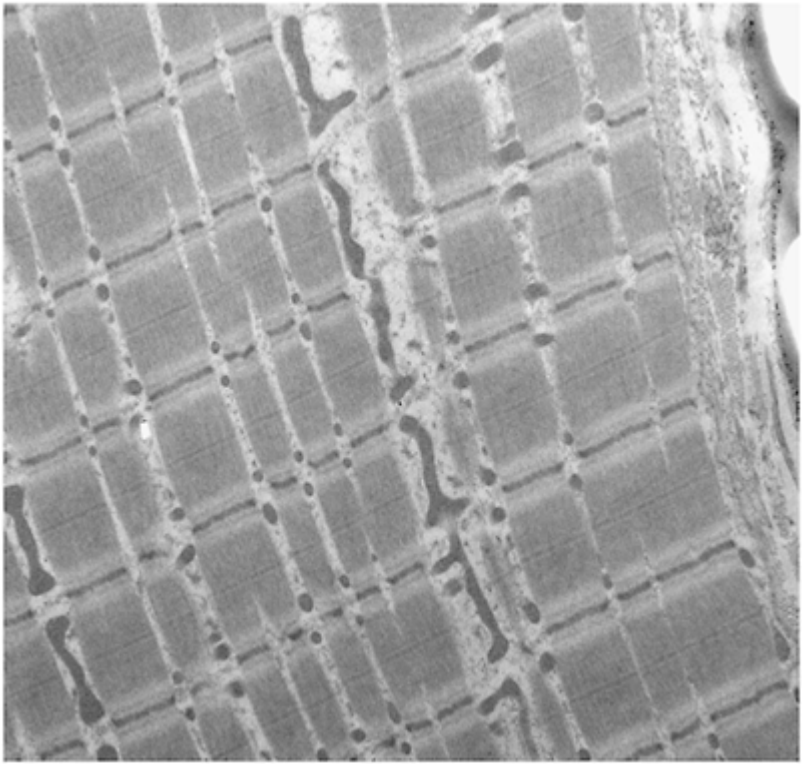

Figure 18.4 Large bizarre shaped mitochondria, probably due to mitochondrial duplication and branching.

1973). In some cases, the mitochondrial clusters are merely a feature of the slow (type I) fibres, and should not be confused with 'ragged-red fibres' the abnormal staining seen on light microscopy that characterize many mitochondrial myopathies (Stadhouders and Sengers, 1987; Sewry and Dubowitz, 2001).

Many of the mitochondria in our cases were irregular and abnormal in shape, and one or two more were darkly stained *(Figure 18.5)* both of which are features of mitochondrial myopathy. Non-specific mitochondrial alterations have previously been described in FSHD (Munsat *et al.*, 1972). Paracrystalline mitochondrial inclusions are a feature of mitochondrial myopathies (Stadhouders and Sengers, 1987). They were described in two of Bethlem's cases, but there was no definite autosomal dominant family history; this paper, however, preceded molecular testing, and the clinical details given are consistent with a mitochondrial disorder (Bethlem *et al.*, 1973). More recently,

but still preceding molecular testing, a family has been described with clinically similar, and overlapping features with FSHD. Paracrystalline inclusions are clearly

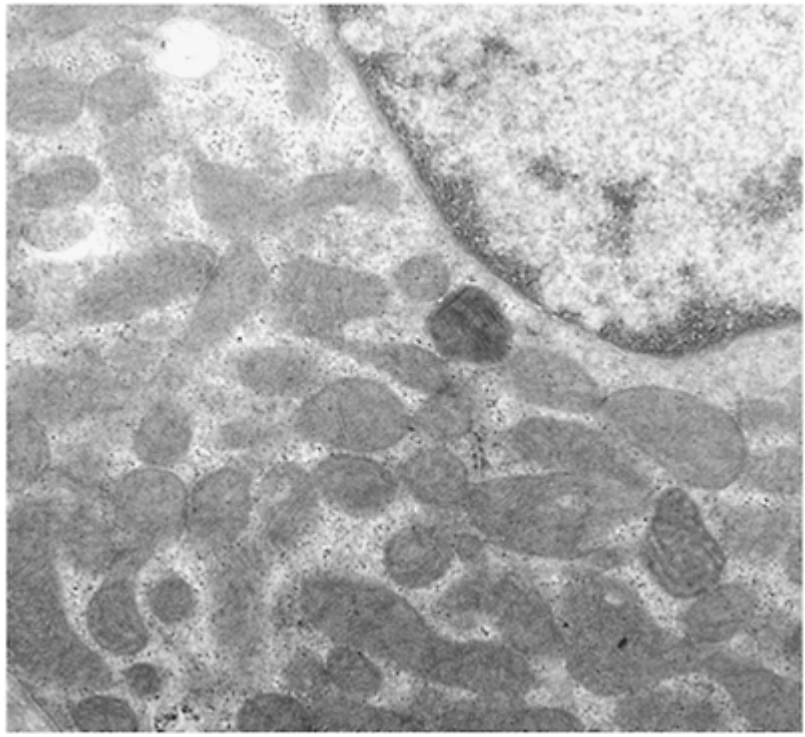

Figure 18.5 Mitochondrial clumping, including a single more darkly stained mitochondrion.

demonstrated in hepatocyte mitochondria, and biochemical tests suggest a defect in complex III of the respiratory electron-transport-chain, due to the absence of cytochrome b. Male-to-male transmission suggests autosomal dominant inheritance (Slipetz *et al.*, 1991). Atypical features and hepatomegaly suggest that this family is not FSHD, or represents a chance association in the one family member extensively investigated. To our knowledge, no paracrystalline inclusions have been reported in molecularly confirmed FSHD.

18.10.3 Basal lamina

A double basal lamina may be due to true duplication, seen in regenerating fibres, or due to redundant basal lamina of an atrophied fibre (Cullen and Landon, 2001). This is also a non-specific feature of many neuromuscular disorders. Duplication of basal lamina was

seen in two cases. The example seen in *Figure 18.6* is most likely related to atrophy rather than regeneration.

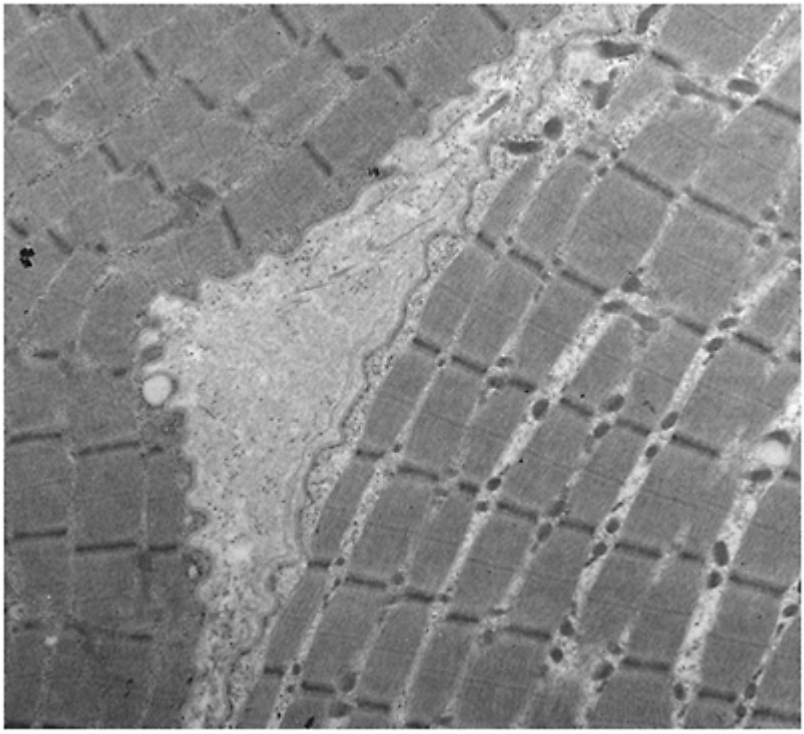

Figure 18.6 A double basal lamina, most likely related to atrophy rather than regeneration.

Perivascular basal lamina

Capillary basement-membrane thickening is a non-specific feature of diseased muscle and can also occur in diabetics. It has been described in polymyositis and was specifically sought for by other authors (Munsat *et al.*, 1972; Bacq *et al.*, 1985). Thickening of the basal lamina of some blood vessels was found infrequently by Munsat *et al.* and was seen in two of the authors' cases (e.g. *Figure 18.7*), but was not associated with perivascular inflammatory changes. The significance of this finding is uncertain. Neither of the authors' cases were diabetic.

18.10.4 Glycogen

Glycogen changes would not be expected in FSHD. Levels of glycogen vary greatly between different fibre types. Although occasional small glycogen deposits were

observed by Munsat *et al.* (1972) and Bethlemet *et al.* (1973), the degree of difference between adjacent fibres seen in one of the authors' cases is unusual. This is likely to be due to a difference in fibre type, but could also be due to loss of myofibrils. Fast-

Figure 18.7 Thickening of the basal lamina in a blood vessel was seen in two patients, its significance is uncertain.

twitch glycolytic (type IIa) fibres tend to contain large reserves of glycogen *(Figure 18.8)*.

Prior to this study, several authors had examined specific facets of FSHD muscle biopsies such as the inflammatory response or the small angular fibres, and others had used FSHD cases as controls for other studies (Munsat *et al.*, 1972; Lin and Nonaka, 1991; Arahata *et al.*, 1988, 1995). In this study, examination of muscle biopsies with a large panel of protein antibodies has found no consistent abnormality across all samples. Small angular fibres are characteristic and common, although not a specific feature of FSHD (Dubowitz, 1985a; Lin and Nonaka, 1991). Thought in the past to indicate denervation, they are also seen in spinal muscular atrophy and motor neuron disease (Lin and Nonaka, 1991). More recently, the small angular fibres have been seen to express fetal myosin in FSHD cases suggesting that they may be regenerating (Sewry and

Dubowitz, 2001). Small angular fibres were not seen in any sample of this study. Why, when apparently so relatively common, they were not seen in any of our biopsies cannot be explained except that sampling differences or choice of biopsy may have played a part. It is unlikely that they were missed because the use of fetal myosin antibody should have made their presence easier to ascertain.

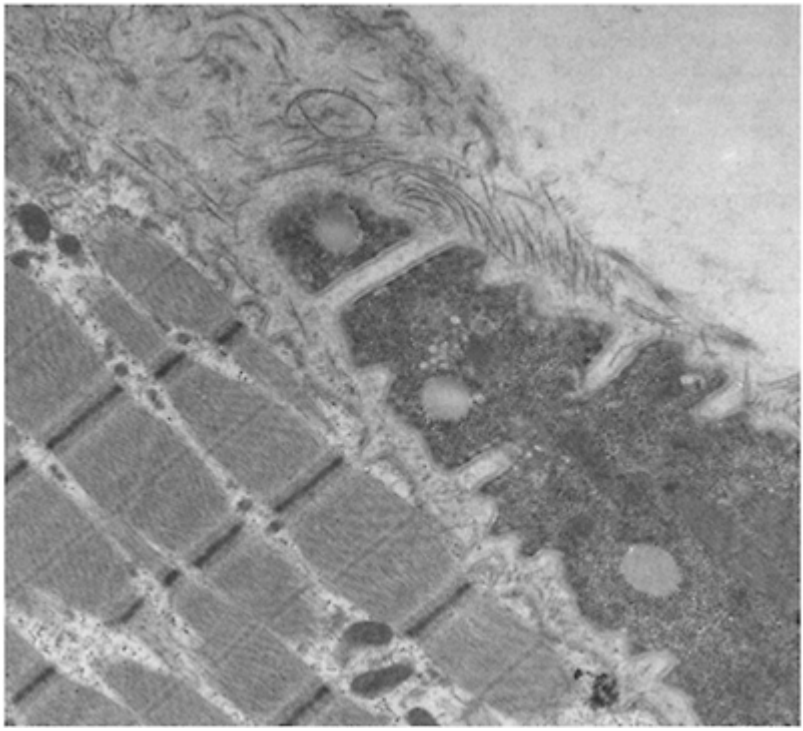

Figure 18.8 Marked difference in glycogen content seen in adjacent fibres.

Having found no unique features which relate to diseased FSHD muscle, it is difficult to know which is likely to be the best avenue for further studies. The small angular fibres, prominent inflammatory element and the suggestion of mitochondrial abnormalities all remain as possible areas for further exploration.

18.11 Conclusion

Current muscle biopsy studies on patients with moderate to severe FSHD including electron microscopy, immunocytochemistry and immunoblotting do not demonstrate any disease-specific, consistent primary or secondary finding. The overall findings are

generally consistent with a myopathic process rather than a neurogenic one. Caspase-3 and fibroblast growth factor expression are likely to reflect non-specific aspects of apoptosis and fibrosis, although a more significant aetiological role for either has not been entirely excluded.

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19.
Linkage analysis in non-chromosome 4-linked FSHD

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19.1 Introduction

Facioscapulohumeral muscular dystrophy is a disease of skeletal muscle, sparing both cardiac and smooth muscle tissues. Its effects include facial and shoulder girdle weakness initially, with progression to involve the pelvic girdle and extremities in the majority of cases. Facioscapulohumeral muscular dystrophy (FSHD) is usually inherited as an autosomal dominant disorder, although sporadic and recessive cases have been described. For nearly all cases of FSHD, the molecular basis of the disease can be identified as a deletion within the D4Z4 repeat at the end of the long arm of chromosome 4. However, in 5–10% of FSHD families, there is no linkage to 4q35. In the small group of families in whom there is no short 4q35 D4Z4 fragment, this type of FSHD is referred to as type 1B (FSHD1B; OMIM 158901) or non-chromosome 4-linked facioscapulohumeral muscular dystrophy.

19.2 Facioscapulohumeral muscular dystrophy is genetically heterogeneous

There have been clues as to disease heterogeneity in previous reports, but until 1993 there were no families identified as being unlinked to chromosome 4q. Linkage studies performed by Wijmenga *et al.* (1991), that sublocalized FSHD to the 4q35-qter region, showed preliminary evidence for heterogeneity with negative lod scores at D4S139 in one small family. During the international effort to locate the FSHD gene, one large family was identified as potentially providing evidence for heterogeneity (Sarfarazi *et al.*, 1992). In addition, an individual with a clear FSHD diagnosis, but with a crossover distal

to D4S139 and D4S163 (the most distal polymorphic 4q markers available at the time) was identified (Sarfarazi *et al.*, 1992) in yet another family.

Gilbert *et al.* (1993) found definitive evidence for heterogeneity in FSHD when a study of seven FSHD families identified two (DUK689 and DUK1361) that appeared to be unlinked to the 4q35 region. Using the markers D4S139, D4S163, and D4S171, multipoint and two-point linkage analysis clearly excluded linkage of these families to the region of 4q. Statistical analysis provided evidence for heterogeneity using two-point linkage analysis, and multipoint analysis supported evidence for heterogeneity, with odds of 20:1 in favour of linkage and heterogeneity when compared to the null hypothesis of linkage and homogeneity. Five of the seven families gave a posterior probability of more than 95% of being linked to 4q, whereas the other two families appeared to be unlinked to this region ($P<0.01$). Both manifested an unequivocal FSHD diagnosis, including facial weakness, clavicular flattening, scapular winging, and proximal muscle weakness. The proband of family 689 was resampled and re-examined, including muscle biopsy. Her clinical signs included scapular winging and neck-flexor weakness; weakness of facial, proximal, and distal muscles was noted. In addition, she had mildly elevated creatine phosphokinase levels. Muscle biopsy findings revealed myopathic changes including rounded atrophic muscle fibers and hypertrophic fibers with occasional internal nuclei. The proband of family 1361 had mild weakness of the orbicularis oris muscles, scapular winging at rest, and moderately flattened clavicles. There was also mild weakness of the deltoids, biceps, triceps and iliopsoas. Exclusion from 4q was further confirmed by fragment size analysis of the D4Z4 repeat with the p13E-11 probe, which revealed that the two non-chromosome 4-linked families did not demonstrate segregation of lower-molecular-weight fragments with the disease. Analysis of the ratio of chromosome 4 to chromosome 10 fragments was 2:2, suggesting no evidence for 4:10 translocation or deletion of the p13E-11 probe binding site. All these observations were consistent with non-linkage to 4q.

In addition to being an important diagnostic tool, the use of the p13E-11 probe to identify FSHD-associated rearrangements has led to further fine mapping of the region on 4q35 and localization to chromosome 4 in small families where power is insufficient to establish linkage with the traditional lod score criterion of 3.0. However, the probe cross-hybridizes to areas on chromosomes Iq12, 3p12, 10cen, and 10q26-qter (Winokur *et al.*, 1994). These regions, which are similar to the chromosome 4-associated fragments, are potential candidate regions for FSHD1B. Gilbert *et al.* (1995) performed linkage studies to investigate the possibility of linkage between the cross-hybridizing regions and non-chromosome 4-linked FSHD. Individuals in family Duke 689 were genotyped for 12 markers, *AMY2B,* D1S185, and *SPTA1* for chromosome Iq12; D3S1217, D3S1284, and D3S1215 for chromosome 3p12; D10S111, D10S174, and D10S196 for chromosome 10cen; and D10S187, D10S216, and D10S212 for chromosome 10q26. All these regions were excluded from linkage, under the assumption that at least two affected individuals for each region had inherited entirely different haplotypes from the affected parent. Multipoint linkage analysis using three markers for each of the candidate regions permitted exclusion of linkage, with lod scores ≤ -2.0 for the 1q, 3p and 10cen regions. Multipoint lod scores for 10q were negative but did not reach standard exclusion criteria. Later work (Speer *et al.*, 1997) has further excluded the telomeric region of 10q as the

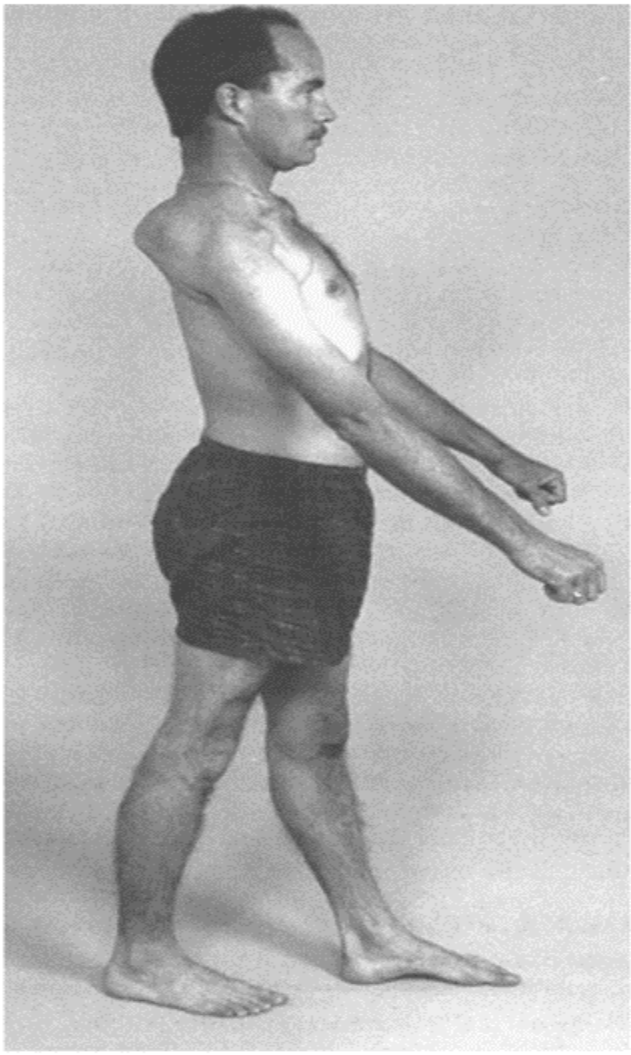
site of the FSHD1B locus. With the use of a more distal marker, two-point analysis was able to exclude ($Z \leq -2.0$) an additional portion of 10q26.

19.3 FSHD1B and FSHD1A are phenotypically indistinguishable

Following the confirmation of genetic heterogeneity, it is imperative to evaluate potential phenotypic differences that could distinguish the types from one another. To that end, Tim *et al.* (2001) clinically characterized affected members of a large, non-chromosome 4-linked family (family 689). Fragment analysis with the p13E-11 probe showed no evidence of short D4Z4 fragment (<35 kb) in multiple affected family members, nor evidence for translocation or deletion, since the chromosome 4 to chromosome 10 ratio is 2:2. Furthermore, cytogenetic studies of the proband were performed to exclude large deletions. At 500-band resolution, studies using fluorescence *in situ* hybridization (FISH) reported the proband to be as karyotypically normal. Several family members were evaluated for facial weakness, scapular winging, muscular asymmetry, and creatine kinase levels. In this large family, 103 subjects were evaluated: 55 (53%) were classified as affected, 18 (17%) as unaffected (no evidence of weakness), and 30 (29%) as of uncertain diagnostic status (mild non-specific weakness or poor subject effort). All of the 55 affected patients had facial weakness, with 41 (76%) having only mild weakness. Scapular winging at rest was noted in 34 patients, and winging with resistance was noted in an additional 15 patients. Asymmetry of the face, scapular, or limb muscles was present in at least 15 of the affected patients and 37 had some degree of hip flexor weakness. Representative photographs of an affected individual in family 689 are shown in *Figure 19.1,* documenting moderate facial weakness, scapular winging, and biceps and triceps atrophy. Serum creatine kinase (CK) levels were measured in 37 individuals, 24 of whom were affected. Only seven affected patients had CK levels above the normal range. The proband showed CK levels 1.4-fold above the normal range and the average for the 24 affected individuals was 1.1x normal, with the maximum being 3.1x normal. In FSHD, serum concentration of creatine kinase is normal to elevated, and usually does not exceed five times the upper limit of the normal range. A muscle biopsy performed on the proband showed atrophic and rounded fibers *(Figure 19.2).* A biopsy on a second affected member of this family revealed no abnormalities other than rare atrophic muscle fibres from light microscopy. Although both myopathic and neurogenic features can be seen in FSHD, the numbers of atrophic fibres were insufficient to allow a diagnosis of neurogenic atrophy to be made. The lack of significant pathological findings upon light microscopy is presumably due to the fact that this was not a clinically weak muscle. A portion of this tissue was used for expression studies, as described later. Thus, this family meets the criteria for FSHD and is clinically indistinguishable from the chromosome 4-linked families.

To date, few FSHD families have been identified as being unlinked to 4q although the exact proportion of unlinked families is still unclear. At this time, four large families have been reported and followed with documented FSHD with no evidence for linkage to 4q (689, 1361, 1392, 2531). In these families, virtually all affected individuals tested for shortened D4Z4 repeat fragments have been documented to have fragment sizes >35 kb. However, occasional individuals within some of these families have been reported to

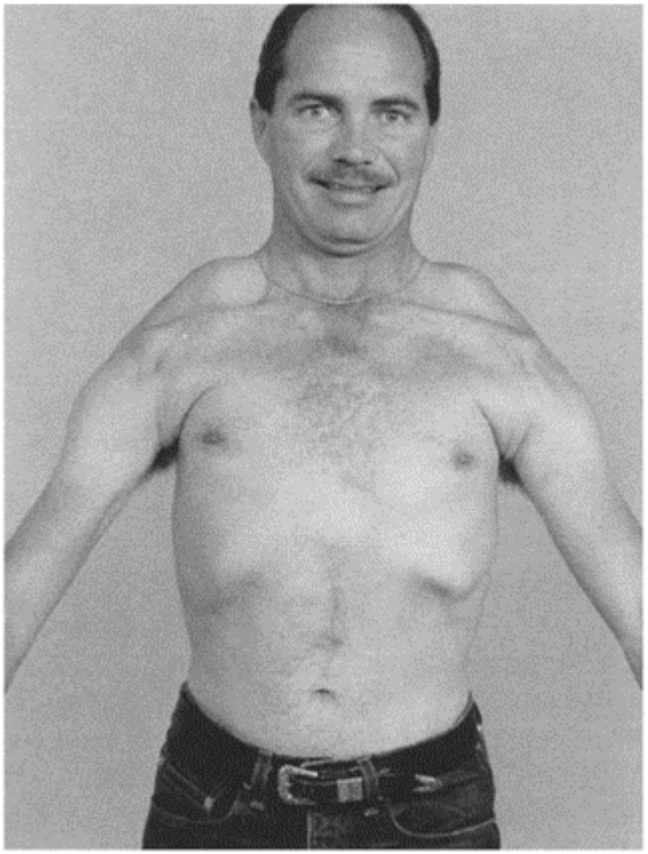
have a small fragment following clinical DNA diagnostic testing. Investigations are currently underway to determine



A

Figure 19.1 Figure (A and B): Thirty-seven year old male, affected for 11 years. Classic features of FSH dystrophy, with moderate facial weakness with marked scapular winging, biceps and triceps atrophy

with relative sparing of the deltoid muscles. Reprinted from Tim *et al.* (2001) Clinical Studies in Non-Chromosome 4-Linked Facioscapulohumeral Muscular Dystrophy. *J Clin Neuromusc Dis* 2001; 3:1–7. With permission from Lippincott Williams and Wilkins, Inc.



B

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whether these findings represent interlaboratory variability or whether they may provide clues as to the pathogenesis of the condition.

19.4 Is FSHD1B allelic to other proximal myopathies?

Scapuloperoneal syndromes, scapuloperoneal muscular dystrophy (SPM; OMIM 181430) and scapuloperoneal spinal muscular dystrophy (SPSMA; OMIM 181405) have similar clinical presentations to FSHD without evidence for facial weakness. Both of these have been previously reported to be linked to chromosome 12 (Isozumi *et al.* 1996; Wilhelmsen *et al.* 1996). Linkage analysis on markers flanking the SPMD and SPMA regions demonstrated exclusion of this region, with lod scores ≤ -2.0 in the non-chromosome 4-linked FSHD families. Clinically, the pattern of weakness and disease onset in the FSHD families were also inconsistent with SPMA (Tim *et al.*, 2001).

Another proximal myopathy is autosomal dominant limb girdle muscular dystrophy. The gene for one of the autosomal dominant forms of this condition has been identified as myotilin (Hauser *et al.*, 2000) and mutations of myotilin have been eliminated as a cause of non-chromosome 4 FSHD (Hauser *et al.*, 2002).

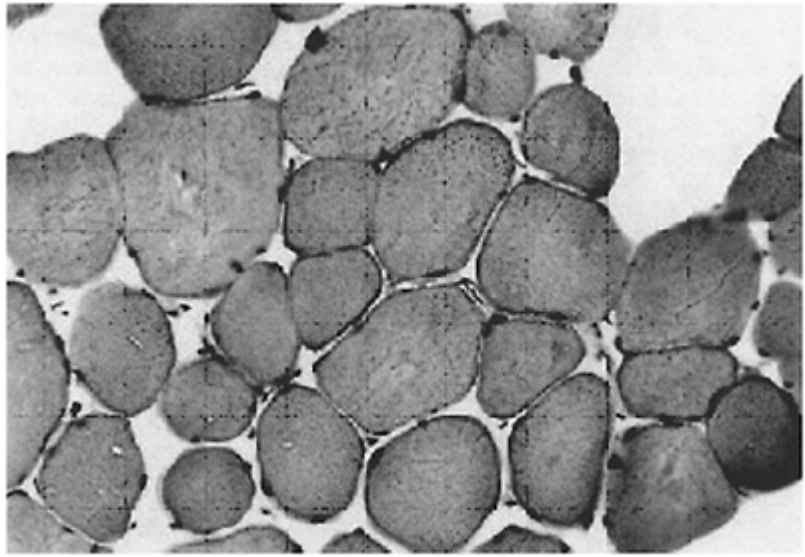

Figure 19.2 Deltoid muscle biopsy of the proband. Haematoxylin and eosin stain (320×). Increased variation of fibre size, rounded fibres consistent with a myopathic process.

19.5 Status of linkage studies in FSHD1B

In families where non-linkage to chromosome 4 has been established, efforts to identify linkage to other areas of the genome have been of limited success. A genomic screen performed on the 4 non-chromosome 4 FSHD families using approximately 430 markers (7–10 cM intervals) is now complete. Family 689, when fully informative has a potential to generate a lod score >16.0. Linkage analysis has, however, failed to identify a significant lod score (unpublished results). These frustrating results can be due to a variety of explanations: diagnostic misclassification, phenocopies within the pedigree, or more than one disease segregating within the family. Alternatively, the genome may not have been fully covered by the polymorphic markers tested to date. Since FSHD1A is telomeric, it is possible that FSHD1B may also represent a difficult-to-identify telomeric locus. Multiple approaches to defining the phenotype and performing the linkage analysis, including both parametric and non-parametric approaches, have been utilized to consider these various hypotheses, again with limited success.

Recently, a region on chromosome 15 that is consistent with linkage (Randolph-Anderson *et al.*, 2002) has been identified in family 1361. An 18 cM region of chromosome 15 provided positive lod score values; multipoint analysis also supported this finding, with peak lod scores reaching 3.20 when one individual, who apparently excludes the region of interest, is eliminated from the analyses. The minimum candidate interval spans D15S1004 and D15S536. In the affected member who appears to exclude this region, analysis of the D4Z4 repeat indicated a length of 30 kb. However, haplotype analysis conclusively eliminated the 4q region in this family and other affected family members display no evidence of a short D4Z4 fragment.

19.6 Candidate gene studies in non-chromosome 4-linked FSHD

Searches for candidate genes in this interval identified the *POLG* gene on 15q25. This gene encodes the gamma subunit of mitochondrial DNA polymerase. *POLG* was an enticing candidate for FSHD1B because it had already been implicated in progressive external ophthalmoplegia (PEO) which involves a mitochondrial myopathy, and more impressively, it has homologues at regions implicated in FSHD, specifically 10q24 and 4q35. However, sequence and mutation analysis of all coding exons and promoter region have suggested that *POLG* is not the gene responsible for FSHD1B in this family (unpublished results). Analysis of the CAG trinucleotide repeat in the coding region of the gene detected no association of repeat length to disease status (unpublished results). Other candidate genes within the region 15q26 have also been evaluated without identification of a causative mutation. These include desmuslin *(DMN)* (an intermediate filament protein that may play a role in muscle integrity) and chromodomain helicase DNA binding protein 2 *(CHD2)* that is possibly involved in the modification of chromatin structure (unpublished results).

Other candidate genes for FSHD1B include those that encode the three, recently identified proteins that bind the D4Z4 repeats of chromosome 4q35. These represent excellent biologically plausible candidate genes. The D4Z4 binding proteins include YY1, a known repressor/activator, HMGB2, involved in the modulation of chromatin

structure, and nucleolin, another transcription factor. These proteins are believed to act as a complex, binding the D4Z4-binding element and acting to repress transcription of nearby genes (Gabellini *et al.*, 2002). It is believed that the derepression of these nearby genes leads to the progression of FSHD.

19.7 Expression studies in FSHD1B

While linkage studies have been the traditional gold standard for identifying regions of interest in the genome that may harbour candidate genes, new methodologies involving identifying changes in gene expression have become available. These approaches allow a complementary method for identifying potential candidate genes under the hypothesis that genes that are either up- or down-regulated in affected tissue when compared to unaffected tissue may be involved in the disease process.

Recently, tissue biopsied from the right quadriceps muscle from an affected patient (described earlier) was processed, and RNA extracted from the muscle tissue sample was tested against RNA from muscle tissue from patients with chromosome 4-linked FSHD and normal muscle tissue. Not only were genes from the non-chromosome 4-linked patient differentially expressed when compared to the normal muscle tissue, but they also demonstrated different patterns of expression when compared to the chromosome 4-linked FSHD patients (Sara Winokur, personal communication). Although preliminary, these results provide further evidence that the pathogenesis of the chromosome 4 and non-chromosome 4 forms of FSHD may be different.

19.8 Summary

Facioscapulohumeral muscular dystrophy is genetically heterogeneous; individuals have been identified in which there is no linkage to chromosome 4q and no short D4Z4 fragment. These FSHD patients are clinically indistinguishable from those individuals where 4q linkage has been established and do not appear to have some other proximal myopathy. Expression studies performed on muscle biopsy from a non-chromosome 4-linked FSHD patient have shown that changes in gene expression may differ from those of chromosome 4-linked patients. Linkage studies in FSHD1B have provided regions of interest that are being followed up and several candidate genes have already been excluded.

The molecular basis of FSHD has been proven to be very complex and to date the FSHD1A gene has not been definitely identified. Mutations found in non-chromosome 4-linked FSH families may shed light on the genetic basis of FSHD1A, the more common type. Efforts to locate the FSHD1B gene have led to few candidates and it is likely that the hunt for the non-chromosome 4 locus will be equally complicated.

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20.
Facioscapulohumeral muscular dystrophy: gender differences and genetic counselling in a complex disorder

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20.1 Introduction

Facioscapulohumeral muscular dystrophy, a genetic disorder with extremely variable clinical severity and age at onset, is classified as an autosomal dominant condition which by definition should affect equally both sexes.

Pedigree analysis has however shown that abortive cases are not rare and that both sexes might not be equally affected. This observation, already made in the premolecular era, has now been confirmed by us at the molecular level. Moreover, the possibility of examining simultaneously patients from two or even three generations and following them in some cases for more than 20 years, has shown that for many affected families, parents are often less severely affected than their offspring, a phenomenon termed *clinical anticipation.*

Although these observations still await a mechanistic explanation at the molecular level, they serve to emphasize the formidable task to be faced in counselling the at-risk relatives of affected patients as well as in prenatal diagnosis.

20.2 Abortive or asymptomatic cases

Landouzy and Dejerine (1885) were the first to describe the most characteristic clinical features of facioscapulohumeral muscular dystrophy (FSHD) and to consider it as a new clinical entity, distinguishing it from the muscular dystrophy previously described by Duchenne. They described a five-generation pedigree where the proband, from the last generation, died of tuberculosis at age 24. When he was 3 years old, atrophy of facial muscles was noted and this was the only symptom until he developed atrophy of the

shoulder girdle and upper arm muscles at age 17. This patient, who manifested the currently termed classical FSHD phenotype, had a brother and a sister similarly affected. According to the description, the proband's father developed muscle atrophy in the shoulder girdle at age 26 with facial involvement at 32, suggesting possible clinical anticipation. The pedigree exhibited a clear pattern of autosomal dominant inheritance but interestingly, the disease appeared to skip the second generation, although individuals from this 'skipped' generation were never clinically examined. Davidenkow (1930) was the first to recognize abortive cases of FSHD and to suggest that this disease might run a milder course in women. Tyler and Stephens (1950) reported the existence of 24 asymptomatic individuals out of 58 patients (48%), 13 of them older than 20 years old. He stressed the fact that often neither the patients nor their families were aware that they had the disease.

Walton and Natrass (1954) defined the pattern of inheritance as being 'usually autosomal dominant, since the occurrence of abortive cases can mask the dominant pattern'. They examined 15 patients and considered five to be abortive (33%), reinforcing the importance of minor facial weakness in the diagnosis and classification of FSHD. However, these authors considered in their studies young individuals who could develop the disease later on. Furthermore, before the availability of molecular analysis, abortive cases could be confirmed only after the birth of at least one affected offspring. In his thesis, Padberg (1982) defined abortive cases as those without symptoms but found to be affected upon clinical examination. The clinical picture could include facial weakness and/or slight shoulder girdle weakness with atrophy.

The great variability in clinical severity and age at onset among patients has always been reported as one of the characteristics of FSHD but it has been generally accepted that the penetrance is around 95% by the age of 30 (Lunt *et al.*, 1989). Considering this, an asymptomatic person would have been someone with no symptoms but with signs of disease that might have been detectable with careful clinical examination, and older than 30 years old.

Only after the probe p13E-11 became available, could healthy 'at-risk' patients' relatives be molecularly tested and confirmed as carriers of the diagnostic small 4q35 fragment. In a first series of 52 Brazilian FSHD families, we observed that 19 of 131 individuals who carried a small *Eco*RI fragment were asymptomatic (Zatz *et al.*, 1998). Subsequently, Ricci *et al.* (1999) reported seven non-penetrant cases with ages varying from 20 to 69 years among 220 unaffected at risk individuals, all of them with fragments ranging from 21–27 kb.

20.3 Gender differences: reports from the premolecular era

Gender differences in FSHD clinical manifestation were pointed out several decades ago. Becker (1953) reported that there were more affected females than males in his study but with no significant difference from the expected 1:1 ratio. Walton (1955) observed that there were 17 affected females and five affected males in four families they studied but considered the sample to be too small to draw any firm conclusions. Chung and Morton (1959) reported that the onset occurred significantly later in males (mean 16.8 years) than in females (mean 13.7 years), suggesting that puberty might play a role in the onset of the

disease. However, this finding was never confirmed by others. By contrast, Davidenkow (1930) suggested that males were more severely affected than females, but only Becker (1953) had evidence for this from his own sample; using pelvic girdle weakness as the criterion for severe involvement, he found significantly fewer women (23%) than men (80%) to be severely affected.

In his thesis, Padberg (1982) reported that the age at onset in the symptomatic group of males and females did not differ significantly. However, if weakness of facial muscles (a sign that often goes unnoticed) is excluded, the mean age of first symptoms occurred significantly earlier in males than in females. Padberg also observed that at age 30, at least 98% of the males but only 81% of the females who will become symptomatic could be diagnosed, suggestive of a lower penetrance in women than in men. In addition, among 107 patients he analysed, a total of 34 (32%) were classified as asymptomatic (without complaints of muscle weakness). However, when gender was considered, Padberg noticed that there were twice as many asymptomatic females (21/48 or 44%) than males (13/59 or 22%). He also pointed out that asymptomatic individuals could be found even in the seventh decade, mostly females.

Later, Padberg *et al.* (1995), in an attempt to determine FSHD prevalence in the Dutch population, put together 97 families: 19 families extensively studied, 30 partially studied and 48 families studied by others. Among the living symptomatic patients, there were 110 males and 103 females. However, when only probands were taken into account, there was a statistically significant excess of males over females (56 and 27, respectively). Since this observation was only found among the probands, they suggested that this distortion was probably due to cultural bias.

20.4 Gender differences following molecular analysis

In a previous study of 52 FSHD Brazilian families where affected and at-risk individuals were molecularly tested, we observed that among symptomatic patients, there was a significantly greater proportion of males (n=56) than females (n=35). The penetrance was estimated to be 95% for males and 69% for females at age 30. This significantly greater penetrance for males than females, although suggested by Padberg in his thesis, could not be proven prior to the availability of molecular testing, possibly because asymptomatic carriers would only be ascertained if they had clinically affected offspring. This was the first time that a significantly greater proportion of females than males, among asymptomatic cases studied at the clinical and molecular level, was described (Zatz *et al.*, 1998). Subsequently, Ricci *et al.* (1999) in a study of 122 Italian FSHD families 78 of which had multiple affected individuals, reported seven non-penetrant gene carriers, six of them females.

In a more recent study in the Netherlands, van der Maarel *et al.* (2000) reported a gender difference among mosaic cases. In a survey of 35 *de novo* FSHD families, based on DNA isolated from peripheral blood lymphocytes, these authors observed that somatic mosaicism (defined as a fifth fragment hybridizing with p13E-1 1) was present in 40% of cases, in either the patient or the asymptomatic parent, with a predominance of females among mosaic individuals (15 females and six males). Interestingly, whilst mosaic males were typically affected, mosaic females were more often the asymptomatic parent of a

non-mosaic *'de novo'* patient. Furthermore, these authors observed that mosaic females had a higher proportion of somatic mosaicism than did mosaic males. Although difficult in practice, it would be extremely interesting to compare the mosaicism rate in different tissues, particularly muscle, from the same individuals.

20.5 Recent analysis of a larger Brazilian sample

In order to confirm our previous observations, we have recently investigated a larger sample of 506 individuals from 106 unrelated Brazilian families with at least one affected FSHD patient (Tonini *et al.,* 2004). The size of the *Eco*RI fragment in asymptomatic or minimally affected carriers as well as symptomatic patients from these FSHD families was measured comparing both genders. In addition, we determined whether asymptomatic carriers were randomly distributed or instead concentrated in some particular genealogies and assessed whether there was preferential parental transmission (maternal or paternal) resulting in non-penetrant carriers. The probands and at-risk relatives were clinically and neurologically examined and the diagnosis was confirmed by molecular analysis. These individuals were classified into three groups according to clinical presentation when last examined.

Group 1

These are asymptomatic or minimally affected (minimal weakness only in upper limbs with or without facial involvement). All individuals in this group were older than 30. Individuals younger than 30 and presenting no symptoms were considered preclinical and not included in this study. This group was created after the observation that some relatives, who were ascertained as being clinically normal, possessed the deleted D4Z4 fragment.

Group 2

This is the classical presentation: mildly affected with weakness only in upper limbs with or without facial involvement or moderately affected with muscle weakness in upper and lower limbs.

Group 3

This group is severely affected: wheelchair confinement or severe childhood form with early onset (symptomatic before age 10).

Molecular analysis using probe p13E-11 revealed that 237 of the 506 tested individuals carried the small *Eco*RI/*Bln*I fragment, with no gender differences (113 males and 124 females). As can be seen in *Table 20.1,* the majority of the patients (n =145, 61%) had the classical phenotype. Although in this group there were more males (n=79) than females (n=66), the difference was not statistically significant (χ^2=1.17; P>0.05). A smaller proportion (17.6%) was severely affected (20 males and 21 females) with a similar distribution for both genders (χ^2=0.09, P>0.05, Id.f.). At the other end of the

spectrum, 51 (21.4%) individuals were asymptomatic or minimally affected carriers. In this last group, there were significantly more females than males (37 females:14 males, χ^2= 10.37, *P*<0.005).

Table 20.1 Analysis of *Eco*RI fragment size and clinical severity in 238 FSHD patients

Clinical severity	N		Mean age		Mean *Eco*RI fragment size			Range
	Males/ Females	Male	Female	Total	Male	Female	Total	
Group 1	14/37	58.8 (15.6)	50.9 (13.7)	53.1 (14.5)	22.6 (4.7)	23.9 (5.3)	23.5[a] (5.1)	12–35
Group 2	79/66	30.8[c] (11.2)	38.7[d] (12.4)	34.4 (12.3)	21.6 (4.9)	22.2 (5.1)	21.9[a,b] (5.0)	12–35
Group 3	20/21	17.2 (16.0)	24.4 (20.2)	21.0 (18.5)	19.8 (5.0)	20.1 (6.3)	20.0[b] (5.7)	10–33
Total	113/124	31.9[c] (16.9)	39.8[d] (16.8)	36.0 (17.3)	21.4 (4.9)	22.4 (5.5)	21.9 (5.2)	10–35

Different letters are assigned to groups with significant differences between means at the 5% level (a and b for fragment size comparison among severity groups; c and d for mean age comparisons between sexes). The numbers in parentheses denote standard deviations.

20.6 Phenotypic severity versus fragment size

The assessment of the size of the *Eco*RI fragment including all patients in the three clinically classified groups confirmed that there is a significant correlation (F= 10.29, *P*<0.005) between fragment size and the severity of the phenotype *(Table 20.1, Figure 20.1)*. Individuals with milder symptoms tend to have the larger fragments whereas those more severely affected have the smaller ones. The same result was observed when fragment size classes are treated as ordinal data (F=4.09, *P*<0.005). When genders are analysed separately, the correlation is significant for females (F=8.76, *P*<0.005). However, surprisingly, no significant correlation between the disease severity and fragment sizes was observed for males (F=1.13, *P*>0.25).

The mean *Eco*RI fragment size was significantly larger in patients from group 1 than group 3 but there was a wide overlap in the distribution: no statistically significant differences were observed between groups 1 and 2 or between groups 2 and 3 *(Table 20.1, Figure 20.1)*. A comparison between families with at least one asymptomatic carrier (*n*=33) and families where all the analysed individuals carrying the FSHD *Eco*RI fragment (*n*=73) were clinically affected, showed that the mean *Eco*RI fragment size was significantly larger in the first than in the second group (23 kb±5.9 and 20 kb±5.75, respectively; *P*<0.05). On the other hand, the mean age at ascertainment including all individuals carrying the small fragment showed that those in group 1 (mean age

53.1±14.5 years old) were significantly older (F=78.6; $P<0.0001$) than those from group 2 (34.4±12.3) who were significantly older than patients from group 3.

The significant correlation between the mean sizes of the *Eco*RI fragment and clinical severity in the total sample observed in the present study is in accordance with previous data, including our two previous studies with Brazilian families as

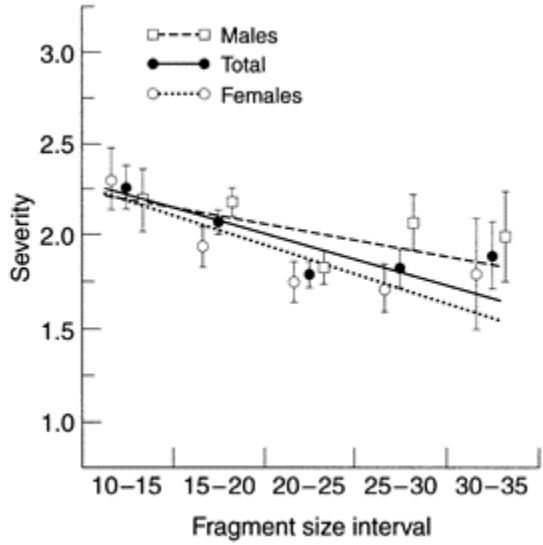

Figure 20.1 Mean clinical severity and mean EcoRI fragment sizes. The correlation between the variables was significant in females ($r=-0.251$, $p<0.005$) and in the total sample ($r=0.225$, $p<0.001$) but not in the male sample ($r=0.162$, $p>0.05$). The lines correspond to the linear regression between fragment sizes and severity of individuals in the considered groups (total, males and female samples). The dots correspond to the means of severity in the intervals of the horizontal axis and the bars represent the standard errors.

well as other population studies (Lunt *et al.*, 1995; Zatz *et al.*, 1995, 1998; Tawil *et al.*, 1996; Ricci *et al.*, 1999). However, when both sexes were analysed separately, the

finding that the correlation was significant for females but not for males was unexpected and still awaits an explanation.

The smallest fragment observed, 10 kb, was noted in an isolated case with the severe infantile form. Fragments of 12 kb were found in two brothers whose mother was a mosaic carrier and also in an asymptomatic female whose father had a classical course (at age 48 he was unable to raise his arms and had facial and lower limbs weakness). It is also noteworthy that on average the smallest fragments were found in isolated as well as mosaic cases. One possible explanation for sporadic cases would be that there is a higher mutation rate from a normal allele to a small fragment size (10–18 kb) than to a larger size (Lunt *et al.*, 1995). However, this observation may be due to an ascertainment bias since isolated as well as the offspring of mosaic parents often have a severe phenotype (Kohler *et al.*, 1996; Tawil *et al.*, 1996; Lunt, 1998; Zatz *et al.*, 1998; Ricci *et al.*, 1999) and it is known that severely affected patients or families with multiple affected relatives are more likely to be ascertained. In addition, patients with a severe phenotype are less likely to reproduce and therefore tend to remain as isolated cases.

Ricci *et al.* (1999) reported in their group of asymptomatic carriers a larger fragment size (ranging from 21 to 27 kb) but their study included only seven non-penetrant cases. On average we observed that the largest fragments were found in the group of asymptomatic carriers but there were many exceptions since small fragments (15 kb and 18 kb respectively) were detected in five (9.8%) patients (three females and two males) with no clinical signs. On the other hand, if there was a correlation between the size of the *Eco*RI fragment and disease severity, an isolated case (or even familial cases) with a large fragment and a mild phenotype would have a greater chance of remaining undetected. Indeed in the present sample, 16 of 52 isolated cases (30.7%) have a severe phenotype while all cases classified in group 1 (asymptomatic or minimally affected) belong to families with at least one clinically affected relative.

In addition, a great clinical variability was seen among patients carrying the same size fragment even within families, ranging from severely affected to asymptomatic. This observation is still more evident in pedigrees with clinical anticipation where the parental generation showed a much milder course than their affected offspring despite carrying apparently the same abnormal fragment.

20.7 Asymptomatic carriers

The proportion of 21.4% asymptomatic or minimally affected individuals found among these 106 Brazilian FSHD families is higher than reported by other authors. One possible explanation is that we have analysed a greater proportion of probands' first degree relatives who were apparently asymptomatic for two reasons: (i) Brazilian families are usually larger than European or North American families; (ii) in our series, there was a greater proportion who were ascertained as apparently isolated cases than in other reports. Indeed, in the publication of Upadhyaya *et al.* (1997), only 27 of 130 families (~20%) were sporadic cases, while in the Italian series of Ricci et al. (1999), of 122 families, 78 had multiple affected individuals while 44 (36%) were isolated cases. Among our families, 51 of 106 (48%) were apparently sporadic cases although after molecular analysis we found 15 families with at least two individuals carrying the FSHD

fragment. Therefore, only 36 of 106 (~34%) were sporadic cases, which is comparable with the observation of Ricci *et al.* in the Italian population. It is noteworthy that Padberg (1982) reported that 30% of the FSHD cases he examined in the premolecular era were asymptomatic, a proportion even higher than the present one.

Another question that was also addressed is whether asymptomatic carriers are randomly distributed or instead concentrated in some families. Although the proportion of families with at least one asymptomatic carrier was 35/106 (32.7%), it was observed that in some families there was apparently a greater proportion of asymptomatic or minimally affected patients. Indeed, 28 of the 51 asymptomatic cases were concentrated in only seven families. Two particularly large families attracted our attention; in the first, although the proband was a 29-year-old male with a classical presentation there were ten individuals (four men and six women) who were classified in group 1 and only two with a classical phenotype among 28 relatives who were tested. In this family, the size of the fragment was 24 kb. In the second family, with a fragment size of 33 kb, even the proband was very mildly affected at age 47, although she had scoliosis since age 13. In this family, six asymptomatic individuals were found to carry the small fragment (including the 72-year-old proband's father) although three of them are younger than age 30 and could still manifest the disease. Interestingly this family was not ascertained based on the FSHD phenotype but because two sibs had another form of muscular dystrophy (Tonini *et al.*, 2002). The observation of some genealogies with multiple asymptomatic carriers supports the hypothesis that a modifier gene, which would protect individuals against the deleterious effect of the FSHD phenotype, would be segregating with a higher frequency in some particular families.

20.8 Parental transmission

It has been reported both by ourselves and others (Zatz *et al.*, 1995; Upadhyaya *et al.*, 1995; Kohler *et al.*, 1996) that severe cases as well as the children of mosaic parents, are either sporadic or more often maternally inherited. Indeed, among 16 of 17 severely affected patients in which it was possible to assess the origin of the mutation, only one was paternally inherited: six were maternally inherited and nine were due to new mutations.

This observation was confirmed in a larger sample since among 31 severely affected cases where it was possible to analyse the parental generation, nine were due to new mutations, 17 (54.8%), were maternally inherited and only five (16.1%) of paternal origin. Interestingly, no statistically significant difference in the mean size of the fragment was observed in the maternally inherited cases as compared to the paternally inherited ones (23.7 kb±4.1 and 26.1 kb±4.7 respectively, $P > 0.22$). Therefore the higher proportion of maternally than paternally inherited severe cases could not be explained by a smaller fragment size in the first group and still awaits an explanation at the molecular level. On the other hand, among 22 cases classified in group 1 where it was possible to determine the origin of the FSHD fragment, 12 were maternally and ten were paternally inherited. This observation supports previous data, which showed that, in contrast to severe cases, there is no preferential transmission for asymptomatic or very mildly affected cases.

20.9 Gender differences

The proportion of males (*n*=113) versus females (*n*=125) who inherited the abnormal fragment did not differ from the expected 1:1 ratio. In addition, the mean size of the *Eco*RI fragment did not differ in males as compared to females in the total sample (21.37 in males and 22.36 in females, *P*>0.05) or in the three severity groups when analysed separately (*P*>0.05).

Among clinically affected patients (groups 2 and 3), even though there were more males (*n*=99) than females (*n*=88), the sex ratio did not differ statistically from the expected 1:1. However, in group 1 (asymptomatic or minimally affected patients), there were significantly more females (*n*=37) than males (*n*=14) supporting our previous observation with a smaller sample and in accordance with some data from the premolecular era (Becker, 1953; Padberg, 1982).

Gender differences have been shown for other autosomal dominant disorders such as myotonic dystrophy (Carey *et al.,* 1994; Gennarelli *et al.,* 1994), Machado-Joseph disease or SCA3 cerebellar ataxia (Ikeuchi *et al.,* 1996; lughetti *et al.,* 1998) and more recently SPG4 spastic paraplegia (Starling *et al.,* 2002). In addition, differences in clinical severity or rate of progression have been suggested for some forms of autosomal recessive limb girdle muscular dystrophy such as telethoninopathy and calpainopathy (de Paula *et al.,* 2002; Zatz *et al.,* 2000, 2003, submitted). The question is whether this difference is due to meiotic drive (that is, preferential transmission of the mutated allele to male rather than female offspring) or to other epigenetic mechanisms that would interact differently with the disease allele according to the gender. Meiotic drive has long been known in *Drosophila*. More recently, a segregation distorter locus encoding a truncated RanGAP (a protein related to the Ran signalling pathway required for nuclear transport and other nuclear functions) has been reported to explain this phenomenon (Kusano *et al.,* 2003). This mutant protein is active but mislocalized to nuclei, which apparently disrupts Ran signalling. According to Merril *et al.* (1999), defective RanGAP would interfere with nuclear transport in spermatids carrying a sensitive responder gene.

For myotonic dystrophy, SCA3 and dentatorubral-palloluysian atrophy (DRPLA), all caused by dynamic mutations, it has been suggested that the excess of affected males could be explained by meiotic drive favouring the transmission of enlarged alleles. SPG4, a pure form of late-onset spastic paraplegia, is caused by mutations in the spastin *(SPG4)* gene. The pathophysiological mechanism has been proposed to be haploinsufftciency with the abnormal phenotype being caused by a 50% reduction in spastin level (Hazan *et al.,* 1999). We have recently identified a large family with 25 affected members but surprisingly only one clinically affected female (although there were 47 men and 45 women in this family). Molecular analysis revealed a significantly greater proportion of men than women who inherited the ‘at risk’ haplotype which is suggestive of meiotic drive. In addition, women carrying the ‘at risk’ allele were not clinically affected. Thus, the paucity of affected females in this family occurred apparently as a consequence of a significant smaller proportion of females carrying the ‘at risk’ allele and additionally by the fact that those who carried it were protected against the deleterious effect of the pathogenic gene mutation. Byrne *et al.* (2000) also reported an Irish family linked to the *SPG4* locus where four of five asymptomatic individuals carrying the at-risk haplotype were females.

For FSHD, we did not observe meiotic drive since the proportion of males and females with the deleted fragment did not differ from the 1:1 expected ratio. Indeed among 238 individuals carrying the FSHD fragment, 113 were males and 125 were females. Thus, the gender difference in clinical manifestation molecularly confirmed again in a larger sample is intriguing. Furthermore, the fact that no significant difference was observed in the mean fragment size in males as compared to females, as well as the lack of correlation between the size of the deletion and the severity of the phenotype in males supports the hypothesis that the gender difference in clinical manifestation in FSHD is the result of another epigenetic mechanism.

20.10 Clinical anticipation

Clinical anticipation was first suggested by our group (Zatz *et al.*, 1995) based on several families where it was possible to examine simultaneously two or even three generations. It was observed that the parents or grandparents were often less affected than their offspring. For 17 multigenerational families, the age at onset as well as age at ascertainment in the affected offspring as compared to those of their affected parents, was analysed. On average, the onset of clinical signs and ascertainment occurred significantly earlier in the affected offspring as compared to their parents, considering all subjects or affected parent/child pairs. In order to verify whether these differences were due to ascertainment bias, the same analysis was performed excluding the index case. The differences were not only confirmed, but were found to be even greater.

Clinical anticipation was confirmed by other authors in distinct populations (e.g. Goto *et al.*, 1995; Lunt *et al.*, 1995; Tawil *et al.*, 1996) although it may not be present in some multigenerational families, as pointed out by Flannigan *et al.* (2001). The problem inherent in such analyses is that data on age at onset of clinical symptoms are not always accurate and consequently may not be reliable. Furthermore, some asymptomatic carriers or minimally affected patients are not even aware that they have some weakness. Therefore, we have recently reassessed the data with a larger number of families. In addition, many of our later-onset patients have been followed for more than 20 years (unpublished observation).

In a set of 47 multigenerational families, the mean age at onset for 45 individuals in the first generation was 38.04±13.61 years whilst the mean age at ascertainment was 53.68±13.96 years. In 63 patients from generation II, the mean age at onset was calculated as 20.4±12.59 and the mean age at ascertainment as 30.31± 13.74 years whereas in 17 patients from generation III, these values were: 17.5± 6.75 and 21.29±6.75 years, respectively. Interestingly, these differences were statistically significant between generations I versus II but not between II and III, suggesting the occurrence of clinical anticipation only between the first two generations. It is important to note that in generation I there is a greater proportion of asymptomatic or minimally affected individuals while in generation II, the majority of cases have a classical clinical presentation. One speculative hypothesis is that the phenotype would be more stable (between two generations) among individuals with the classical phenotype than among the asymptomatic or minimally affected ones who would have a greater chance to have symptomatic rather than asymptomatic offspring. This observation, if confirmed, would

also explain why anticipation is not found in some genealogies where there are many individuals with the classical form of FSHD.

When the ages at onset and ascertainment were analysed within each family, we observed that clinical anticipation occurred in 70% of the families (33/47) and in 63.7% (30/47) among them when asymptomatic mosaic carriers were excluded. Therefore, although anticipation occurred in most of our families, it was not present in about 30% of the pedigrees, in accordance with the observation reported by Flanningan *et al.* (2001) in a multigenerational Utah FSHD family.

20.11 Is clinical anticipation influenced by the gender of the transmitting parent?

One very interesting family attracted our attention with regard to another aspect. In this family, the 20-year-old proband is an isolated severely affected male, symptomatic since the age of 1 year. His 55-year-old mother is completely asymptomatic at clinical and neurological examination. Molecular analysis revealed that the mother carried the same-sized *Eco*RI fragment present in the proband which was also found in his older 33-year-old unaffected sister. We had previously observed that among 27 offspring born from 15 asymptomatic mothers, there were significantly more affected sons (20) than daughters (seven) (Zatz *et al.*, 1998). This finding together with the present family raised the question as to whether clinical anticipation might not only differ among sibs but might also be influenced by the transmitting parent and/or offspring gender. In order to test this hypothesis in a larger sample, we compared four possible transmissions: mother-son, mother-daughter, father-son and father-daughter. The observed frequency of clinical anticipation was: 38/45 (84.4%) for mother-son transmissions; 21/31 (67.7%) for mother-daughter transmissions; 8/16 (50%) for father-son transmissions and 13/19 (68.4%) for father-daughter transmissions. Therefore, although it occurred in all groups, clinical anticipation was significantly more frequent for mother-son transmissions. It is important to point out that this observation, which has important implications in genetic counselling, had previously been reported for other genetic disorders such as myotonic dystrophy (Brunner *et al.*, 1993; Zatz *et al.*, 1997).

20.12 How to relate clinical and genealogical observations and new molecular findings

Van Geel *et al.* (2002) reported the existence of a polymorphic segment of 10 kb distal to D4Z4, with alleles 4qA and 4qB. Lemmers *et al.* (2002), in a survey in the Dutch population, evidenced the importance of these finding to FSHD diagnosis. Analysing 80 control individuals and 80 unrelated FSHD patients, they observed an almost equal frequency of 4qA and 4qB alleles in the first group. However, the FSHD allele was exclusively of the 4qA type. Moreover, when the parental normal-sized ancestor of the FSHD allele was available for study, it was seen that it was always from the 4qA type. In six individuals with non-pathogenic *'de novo'* deletions, both 4qA and 4qB alleles were rearranged, suggesting no difference in their propensity to contract. Therefore, there

should be a functional difference between 4qA and 4qB alleles. Although we have not determined in Brazilian families the 4qA and 4qB nature of the deleted allele, we observed that the unaffected relative always had the same-sized deleted fragment as the patient. Thus, it is very unlikely that the so-called 'non-manifesting' relative would carry a 4qB-type fragment of exactly the same size as the FSHD allele.

The transcriptional derepression model proposed by Gabellini *et al.* (2002), where genes at 4q35 are overexpressed in a manner inversely related to distance from D4Z4 and D4Z4 repeat number, would explain why females carrying larger deletions are more severely affected. However, it would not explain the clinical variability observed in FSHD males if the lack of correlation between the size of the deleted fragment and the course of the disease, observed by us, were to be confirmed in another studies.

In addition, the observed intrafamilial clinical variability in patients carrying the same-sized deleted fragment, the occurrence of clinical anticipation in most multigeneration families, the gender differences in clinical manifestation and transmission of the abnormal allele as well as the fact that a proportion of cases remain asymptomatic are still awaiting an explanation.

20.13 Genetic counselling and prenatal diagnosis

Counselling FSHD families, taking into account all these variables, is not an easy task. The impossibility of predicting the severity of the disease in fetuses who carry the FSHD allele or in future offspring as well as issues such as clinical anticipation, gender differences and asymptomatic carriers are discussed during genetic counselling (GC) with couples who plan to have children. It has been our policy not to test young 'at-risk' asymptomatic children even if the parents request it. The main reason is that since no prognosis can be established, the impact of 'knowing' that you may carry a 'disease allele' would probably be more detrimental than beneficial. In addition, if we test a child, we are denying them the right to decide later as an adult, whether they want to be submitted to molecular testing or not.

On the other hand, revealing carrier status to an adult clinically unaffected relative is also a complicated ethical issue. If the asymptomatic carrier is too old to have children, there is no benefit to be obtained in communicating such information. However, in the case of young adults, they should be informed that the risk of transmitting the deleted allele to the offspring is 50%. Moreover, although no prediction can be made as to the clinical course of the disease in future children, the possibility of clinical anticipation should also be discussed according to the gender of the transmitting parent and offspring. For example, if we take into consideration our observation in FSHD Brazilian families, we should alert a minimally affected female that the chance that her children may have a more severe course than herself is greater than 65%.

Even more difficult is the issue of prenatal diagnosis and the decision whether or not to terminate a pregnancy if the fetus is found to be carrier of the *Eco*RI deleted allele. On the one hand, if the fetus is a female, she will have a greater chance to have a milder course or even to remain asymptomatic, than if it is a male fetus. On the other hand, the possibility of clinical anticipation has also to be discussed in particular when the

consultant affected parent belongs to a family where clinical anticipation has been observed and according to the sex of the transmitting parent and child.

Counselling couples, who come for prenatal diagnosis, also bring different dilemmas according to whom is the affected parent in the 'pregnant couple'. If the mother is the 'affected' member, she can always say that she does not want a future child to suffer based on her own life experience. However, the situation may be more complicated when the father of the fetus is the affected one. For example, one couple referred to us for prenatal diagnosis because the husband had FSHD decided afterwards that they would not be tested because the pregnant mother felt she would not mind having a child with the same disorder as her husband. However, if the mother were to decide that she wanted to interrupt the pregnancy because the fetus had the same disease as her husband, would this not imply that she would have aborted her own husband or partner?

On the other hand, our experience has shown that the patient's own perception and observation, particularly for those belonging to families with multiple affected patients, plays a fundamental role in decision-making. This can be illustrated in the two situations described below, where prenatal diagnosis had identified for both cases female fetuses carrying the abnormal FSHD fragment. In case 1, the consultant mother was severely affected at age 43, when she became accidentally pregnant. When submitted to prenatal diagnosis, she was unable to raise her arms and could walk only with support. When informed of the molecular results, she decided to terminate the pregnancy, a decision based mainly on her own fear of having a child that could be more affected than herself since her phenotype was much more severe than that observed in her own mother, who was in her sixties. Clinical anticipation in her family helped her to take this decision.

In case 2, the affected parent was the father who had a classical phenotype (facial, shoulder and upper arm weakness) at age 31. When prenatal diagnosis was requested, the expectant parents had not yet decided whether or not they would terminate the pregnancy in the case of an affected fetus. However, when they were informed that the fetus was a female, they decided to continue the pregnancy based on their own perception that women had a greater chance of having mild disease. Indeed, the mother of the affected father was very mildly affected at age 55 and he had an older sister who was asymptomatic at age 33.

In summary, until a cure is available for FSHD, genetic counsellors should be prepared to discuss all these issues with affected families trying always to present the positive and negative impact of available information in an even-handed way.

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21.
Genetic counselling for facioscapulohumeral muscular dystrophy (FSHD)

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FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

21.1 Introduction

Genetic consultation will be requested when a person themselves, their partner, child or other relative is believed to be affected with FSHD. The first consideration is whether the diagnosis is correct, and if so, whether this has been confirmed at a molecular level. The molecular diagnosis of FSHD is discussed in Chapter 15.

Personal experience from a diagnostic laboratory (Bristol, UK) receiving DNA samples from many different sources (mainly neurologists and geneticists) for testing for FSHD, suggests that in most cases of definite FSHD it is possible to confirm the diagnosis by demonstrating a typical molecular result, but that exclusion of the diagnosis in non-FSHD cases is often much harder, particularly if only a shortened *Bln*I-sensitive DNA fragment is present. An additional technique, a *Bgl*II/*Bln*I dosage test (van der Maarel *et al.*, 1999), is helping to exclude effectively the diagnosis from some of these samples with *Bln*-sensitive fragments (converting some probable negatives to more certain negatives), but does not significantly reduce false positives nor false negatives, and hence cannot usually establish the diagnosis in those *bona fida* cases that do not have a standard molecular result (i.e. the test does not really improve sensitivity).

Very recently, a telomere polymorphism identified distal to the D4Z4 repeats at 4q35 (Lemmers *et al.*, 2002) has suggested an absolute requirement for association with a type A polymorphic allele to be in *cis* for D4Z4 deletion to cause FSHD (Lemmers *et al.*, 2003; van der Maarel *et al.*, 2003). Testing for this polymorphism may now help to reduce some false positives (when the telomeric polymorphism is a type 4 B allele) or false negatives (when the p13E-11 probe locus is deleted).

The latter may slightly improve sensitivity, but the main problems in interpretation of diagnostic testing will remain:

1. The finding of a shortened *Bln*I-sensitive repeat in someone where a clinical diagnosis of FSHD is quite possible but not certain.
2. The finding of a *Bln*I-resistant repeat at the upper end of the size range (i.e. 35–42 kb, and therefore in the range of overlap with normal controls) in someone whose clinical presentation would be rather atypical for FSHD.

Accordingly, the consideration of genetic counselling issues (below) is for application only to families (or isolated cases) where the diagnosis is already proven in a clinically affected case.

Requirements for family testing

For confirmation of diagnosis at the molecular level, an essential prerequisite for any molecular prediction, the DNA must show a deletion of some of the 3.3 kb repeat units at 4q35 on one copy of chromosome 4, such that the residual repeat length is between one and 12 copies (van Deutekom *et al.*, 1993; Butz *et al.*, 2003). The possibility also of an absolute requirement for association with a type A polymorphism at the telomere of 4q may additionally help identify the disease-associated D4Z4 fragment in patients who appear to have two different BlnI-resistant shortened *Eco*RI fragments (van der Maarel *et al.*, 2003). If no sample is available from an affected person or relative, genetic counselling can initially only really be given provisionally, according to empirical clinical criteria, and definitive advice should only follow after DNA testing of the consultand.

With a confirmed diagnosis, genetic counselling then depends on application of genotype-phenotype correlations as well as the basic pedigree structure. For this, it is essential (where possible) to know the size of residual fragment on 4q, whether the mutation appears to be familial or *de novo* (i.e. not found in either parent), and whether it may be present as a somatic mosaic.

Counselling issues can then be addressed according to the situation and question. Broadly, FSHD cases and families can be divided into three types (types I, II, III)(Lunt et al., 1995a, 1995b):

I. Isolated cases, usually from *de novo* mutation, usually severe onset, and small residual fragment size of <18 kb.
II. Typical classic cases, often have extensive known family history but pedigree may include occasional asymptomatic obligate heterozygote; fragment size in middle of range (18–30 kb). It is from this group that most of the broad counselling data have been derived (Lunt and Harper, 1991).
III. Milder cases, later onset, and may present with scapular weakness alone (Jardine *et al.*, 1994a). Relatively large fragment size (30–40 kb). Often there is only a minimal family history apparent, but further investigation reveals a much more extensive family history of heterozygous carriers for the mutation, many of whom are clinically asymptomatic and with no positive clinical signs. *De novo* mutation is exceptional (Upadhyaya *et al.*, 1993; Lunt *et al.*, 1995a; Butz *et al.*, 2003; Tonini *et al.*, 2003).

21.2 What are the common genetic situations and questions encountered in the genetic clinic in relation to FSHD?

21.2.1 Patient themselves affected with FSHD (or affected child)

Treatments

1. What can I do to improve muscle strength (mostly for elevation of the arms)?

2. What can I do to improve body image and self-esteem (especially with wasted upper arms or pectorals in young men)?

3. What can I do to reduce muscle pain and discomfort (especially around the shoulder girdle)?

The clinical questions on treatments are primarily addressed in other chapters. Generally it is felt that regular gentle to moderate exercise is beneficial, but that hard exercise or weight-training could be likely to accelerate muscle damage. Most patients show asymmetric involvement of shoulder girdle and upper arm muscles, in which the dominant side (usually the right side) tends to be affected first and to the greatest degree (Lunt and Harper, 1991). This, together with an observed often irreversible muscle wasting of a limb following enforced immobility (e.g. secondary to a fracture) (Padberg, 1982), has suggested that the underlying impairment in FSHD might affect regeneration of damaged muscle. Hence, hard exercise may be inadvisable. Hydrotherapy or swimming may be helpful alternatives in allowing exercise without concomitant weight-bearing.

There is no specific therapy for FSHD. Results of therapeutic trials (e.g. with steroids, beta-agonists, vitamin E) have not led to any consistent improvements or recommendations (Tawil *et al.*, 1997; Kissel *et al.*, 1998; Padberg, 1998). From anecdotal experience, some individual patients have found ‘alternative therapies’ to have helped them with their condition, particularly in relation to muscle pain and discomfort. In some people this aspect can be a greater problem than muscle weakness. There is no specific recommended analgesic agent, although empirically, non-steroidal anti-inflammatory drugs might be the first ones to try, given that muscle biopsy in FSHD in some patients can show a relatively prominent inflammatory component (Padberg, 1998).

Surgery, and particularly scapular fixation, has been helpful in some patients in enabling greater use of the arms, although this does mean that the arm cannot then be raised above shoulder level (Twyman *et al.*, 1996; Padberg, 1998).

Prognosis

Will I/they become disabled?

FSHD is a progressive condition, but almost always with facial and shoulder girdle involvement preceding lower limb or distal upper limb involvement. Onset of symptoms of FSHD, particularly in the shoulder girdle, will usually be asymmetric, but will usually

be followed eventually by involvement of the opposite side. Some patients can have periods of plateauing of muscle weakness lasting many years or even decades, but there can often be periods of more rapid progression, particularly in late teenage years or early adulthood (Lunt and Harper, 1991). Anecdotally, several patients have described their awareness of foot drop as seeming to come on acutely, while some women have commented that they have experienced more rapid progression of pelvic girdle and proximal lower limb weakness following a pregnancy.

The prognosis in an individual patient is subject to the overall broad inverse correlation between severity and residual D4Z4 repeat number, as discussed in more detail below. It is also now apparent that males tend to have slightly earlier onset than females and also tend to have a greater severity of involvement, at least up to age 50–60 years (Zatz *et al.*, 1998); one earlier study suggesting a mean onset age of 15.8 years in males, but 19.0 years in females (Padberg, 1982).

One of the main concerns for a patient may be to know whether they will progress to require a wheelchair. For those patients with the smallest number of residual repeats (1–3 repeats), who will also almost invariably have proven *de novo* mutation, or who will have one parent mosaic for the mutation, the likelihood of loss of ambulation is high, and this has first occurred by age 8 years up to age 25 years (Lunt *et al.*, 1995a). At the upper end of residual repeat numbers (9–12 repeats), the risk is likely to be very small. Data from typical familial cases in large families (with approximately 4–8 residual repeats), found 20% of patients aged between 40 and 83 years to require a wheelchair, but all patients in this group had in retrospect been aware of some lower limb weakness by age 20 years (Lunt and Harper, 1991). Conversely, if there is no proximal lower limb involvement by the early 20s, the likelihood of eventual requirement for a wheelchair by age 50–60 years is small.

21.2.2 Patient themselves affected with FSHD, or affected child or relative

Genetic questions

1. Is my child at risk?

2. If so, what is the likely severity?

3. Is there a difference between a boy or girl?

4. Can the condition be avoided—i.e. is there a prenatal test (or preimplantation testing)?

5. Where did the condition come from?

6. Am I at risk (if a relative is the one affected)?

Specifically different genetic situations encountered

1. Typical classic familial case—known affected.
2. Clinically unaffected but with known typical dominant family history.
3. Mild presentation with or without a known family history:

   a person themselves affected;
   b clinically asymptomatic, but relative affected.

4. Severe case in a relative:

   a in a sib;
   b in another relative.

5. Parents of a severely affected child:

   a same set of parents;
   b new partner.

6. Severe case in consultand.

21.3 Specific genetic situations

21.3.1 Typical classic familial case—known affected

Is my child at risk?

FSHD always follows autosomal dominant inheritance. Even an isolated case known personally to the author where parents have been consanguineous, has proved on molecular testing to have a typical *de novo* 4q35 D4Z4 deletion. The genetic risk to offspring of someone with FSHD is therefore always 50% (1 in 2). If both parents are affected, and there is a potential (1 in 4 risk) for a child to be conceived as a homozygote for FSHD, the clinical presentation of homozygous FSHD is not yet known.

Given that my child is at (50%) genetic risk, what is the likely clinical severity? Is there a difference between a boy or girl?

Clinical severity has been defined in different ways in different studies according to the parameters chosen to assess this. These parameters have included:

1. Age at onset of identifiable clinical signs, or retrospective onset of clinical symptoms (Lunt *et al.*, 1995a; Zatz *et al.*, 1995).
2. Simple overall 3-point or 5-point severity grading combining MRC-scaled assessment of muscle strength, particularly in lower limbs, with current functional disability (Lunt and Harper, 1991; Zatz *et al.*, 1998; Ricci *et al.*, 1999; *Butz et al.*, 2003).
3. Myometry across several different muscle groups, combining a maximum voluntary isometric contraction test, with manual muscle testing (Personius *et al.*, 1994; Tawil *et al.*, 1997; Rogers *et al.*, 2002).

Severity in offspring may vary according to:

1. fragment size (which determines a particular range of severity);
2. sex of offspring;
3. suggested possibility of clinical anticipation;
4. other factors.

For a typical familial case, the mutation causing the FSHD will necessarily be constitutional (present in all cells), unless the person is the first one recognized in the family to have symptoms, in which case it could possibly be mosaic (van der Maarel *et al.,* 2000). In a pedigree with several known affected members, the range of severity averaged across these will give the best guide. In typical large families, overall penetrance for clinical signs has been estimated at 95% beyond age 20 years (Lunt *et al.,* 1989). There is currently increasing evidence that onset in females tends to be at a later age, and to be of lesser severity than in males in the same family (Zatz *et al.,* 1998). Non-penetrance is more likely in women than men; penetrance at age 30 years has been re-estimated in another study as 95% for men, but only 69% for women (Zatz *et al.,* 1998). Thus, sons of affected mothers tend to show the biggest shift in increase of severity, whereas daughters of affected fathers may show the least. However, it is rare for a child to be affected to a lesser degree (later age at onset) than the affected parent, even the daughter of an affected father.

Several researchers (Lunt *et al.,* 1995b; Zatz *et al.,* 1995; Tawil *et al.,* 1996) have suggested that there might be clinical anticipation operating, even though the mutation within a family seems, in all but very exceptional cases (Lemmers *et al.,* 1998), to remain the same. The prevalence of a *de novo* mutation occurring as a somatic (at least 40% of cases) rather than a germline mutation, and hence giving rise to mosaicism and consequent milder clinical presentation, may help to account for apparent anticipation which cannot be accounted for by any acertainment bias alone (van der Maarel *et al.,* 2000). However, this debate continues.

The overall data for a typical classic familial case are summarized below (Padberg, 1982; Lunt and Harper, 1991).

Onset age

First symptomatic onset is typically from 8 years to 25 years and often in the second half of the second decade (mid- to late teenage) presenting with scapular winging, humeral wasting or scapular girdle weakness. However, 50% show recognizable clinical signs (usually as peri-oral and/or peri-orbital weakness by 11 years), and 95% by age 20 years (Lunt and Harper, 1991). Penetrance is age-dependent, but there may be up to 30% of females who remain asymptomatic beyond 20 years of age (Zatz et al., 1998).

Severity

Around 20% of cases may become severe enough to require a wheelchair beyond 40 years of age, but all these would be expected to show evidence of lower limb weakness by their early 20s (Lunt and Harper, 1991).

Can the condition be avoided—i.e. is there a prenatal test (or preimplantation testing)?

Prenatal diagnosis by DNA analysis from chorion villus biopsy (CVS) usually taken at 11 weeks gestation will be possible where the affected parent has a standard D4Z4 deletion with a defined recognizable residual *Eco*RI /*Bln*I DNA fragment (Bakker *et al.*, 1996). It is recommended that DNA from the partner should be analysed at the same time, to check that there are no potentially confounding chromosome 10 fragments that could lead to possible confusion in interpretation of the result. The size of residual fragment is such that analysis of CVS DNA will require Southern blotting rather than PCR, and a sufficient CVS sample should be taken to enable this. Analysis should include *Eco*RI and *Bln*I digest, since there are some affected patients who can have both a *Bln*I-resistant and *Bln*I-sensitive fragment of the same size. Rarely a patient may have two *Bln*I-resistant fragments of the same size (one on chromosome 10, as well as the FSHD-causing one on chromosome 4). In such cases, or if there could be confusion between two *Bln*I-resistant fragments differing in size by (say) only 3 kb, it would be wise in familial cases to combine the direct test for the D4Z4 residual fragments with analysis of linked DNA markers (such as pH30 at D4S139 for 4q35, and markers at D10S590 and D10S212 for 10q26) by including other affected family members and their spouses (especially both parents of the consultand if possible) (Bakker *et al.*, 1996). In addition, use of the telomeric A/B polymorphic probe may determine which of two *Bln*I-sensitive fragments is associated with FSHD (van der Maarel *et al.*, 2003).

At present it is not possible to offer preimplantation genetic diagnosis except by linkage as testing for the mutation itself would require PCR techniques to be applicable.

Where did the condition come from?

In typical familial cases, this will be self-evident. A new mutation will always have occurred somewhere generations further back in the family. The likelihood of being able to identify the original new mutation in a family reduces with increase in fragment size (Lunt *et al.*, 1995a). Most cases with the smallest number of residual D4Z4 repeats (7–15 kb residual fragment), are new mutations; whereas with fragments >30 kb it is likely that there will be an extensive family history but with many asymptomatic heterozygotes. For families where sibs are affected, but neither parent shows the mutation, it is likely that one of the parents will be a germline mosaic, or perhaps a very low level somatic mosaic (Upadhyaya *et al.*, 1995; Kohler *et al.*, 1996; van der Maarel *et al.*, 2000).

21.3.2 Typical dominant family history: consultand themselves clinically unaffected

Is my child at risk? Am I at risk (if a relative is the one affected)?

All the same questions as above depend first on whether the person has or has not inherited the D4Z4 deletion causing the FSHD in the family. They should however first be assessed by clinical examination. The most minimal (and usually the earliest) clinical signs are most often seen in facial muscles (Padberg, 1982, Lunt and Harper, 1991).

Specifically there may be peri-oral weakness, usually asymmetric, demonstrated by asking the patient to whistle, blow, smile, pout, and by testing the strength of cheek puff against closed lips; or weakness of eye closure. This is best tested by asking the patient to close their eyes fully, and by observing whether the eyelashes can be buried, and how much force is required by the examiner to prise open the eyelids. Significant asymmetry in the shoulder contour due to a 'dropped' shoulder or over-riding scapula, increased scapular winging, or detectable muscle weakness in the shoulder girdle and periscapular muscles may be evident, or possibly weakness for ankle dorsiflexion.

If the person is asymptomatic and has no clinical sign to suggest FSHD, one may be able to give some measure of reassurance from the normal examination alone. However, this can only really be offered if:

1. they are already beyond the age at which the latest onset of symptoms has occurred in affected members of the family.
2. there is no likely case of non-penetrance in the family.
3. all affected members have had a relatively severe presentation.

If they would be at 25% prior risk from the pedigree structure, their own risk will of course become very small if their parent satisfies the above criteria, but history alone cannot be used to assume that a parent is unaffected.

For DNA analysis, a DNA sample from a known affected family member should first be obtained and tested to confirm the diagnosis at the molecular level, and to define the mutation causing the FSHD. DNA from the consultand can then be tested against this to show whether or not they have inherited the mutation.

If no DNA sample can be tested from an affected family member, interpretation of the DNA result for the consultand in isolation may be hazardous. The prior chance of the family having a typical D4Z4 deletion must first be estimated from the clinical history of affected family members. For typical large families with many affected members, this will be high (i.e. >92%) (Lunt, 1998). However, it is more likely that the uncertain situation will be encountered in families where the number of known clinically affected family members is small, either due to the presentation being severe and limiting reproductive fitness, or the presentation being mild and clinically detected in few. Increasingly, family separation may also limit information and access to wider family members.

In these circumstances, the finding of a typical *Bln*I-resistant fragment will strongly indicate that the consultand has inherited the condition provided that the D4Z4 fragment size is satisfactorily in line with the broad overall correlation of severity with fragment size, both for presence or absence of clinical signs in them, and for the history in their affected relatives. Thus, for example, a *Bln*I-resistant fragment of 30–38 kb would be compatible with a relatively mild presentation in relatives and minimal or no signs in the consultand. However, a *Bln*I-resistant fragment of 35–40 kb in the context of a typical moderate to severe presentation in the family history would be more likely to indicate a coincidental fragment, whether on 4q35 or 10q26 and unlikely to indicate that the person would have inherited the family condition.

When there is no DNA sample from an affected family member, the finding of a *Bln*I-sensitive fragment poses even greater problems, and unless the size is well outwith the fragment size expected from the clinical history, or can be proved by some method to be

on chromosome 10, or conversely proved to be on chromosome 4, the genetic status of the consultand cannot be satisfactorily resolved, as it is not then possible respectively to exclude or confirm the diagnosis.

A table of likelihood of someone having FSHD according to their DNA result, by fragment size, *Bln*I-sensitivity/resistance, and dosage of *Bln*I-resistant: *Bln*I-sensitive fragments has been attempted on a theoretical basis (Lunt, 2000), reproduced here as *Table 21.1*. For the situation of an apparently asymptomatic consultand, a likelihood value can be read from this table which can then be applied in a Bayesian calculation to an estimated prior risk for them having FSHD on clinical grounds. It is this prior risk which is hardest to estimate as it requires consideration of age of onset factors as above, combined with straight pedigree risk.

Clinical advice following confirmation of 4q35 DNA fragment in clinically unaffected or minimally affected person from a known FSHD family

For the person themselves, guidance for their own future prognosis comes from the combination of:

1. Current clinical signs and symptoms (if any) in relation to their current age.
2. Range of age at onset and clinical course in other affected family members.
3. Sex (males tend to present earlier and have a more severe course than females; a higher proportion of females can remain asymptomatic).
4. D4Z4 fragment size.
5. Other coincidental conditions or lifestyle factors.
6. Possibility of clinical anticipation (less likely to need consideration if person is asymptomatic).

FSHD is invariably a progressive condition. However, there can be long periods of plateau in relation to this. Overall, as a general guide, if someone does not exhibit any lower limb weakness by their early 20s they are very unlikely to progress to requirement for a wheelchair by 40–50 years and possibly not at all. This observation was derived from data on large families, where amongst 35 affected subjects over 40 years who were classed as ‘severely affected’ 34/35 had by history been aware of lower limb weakness by their early 20s (Lunt and Harper, 1991).

For offspring of the person, who are then confirmed to be at 50% risk, guidance regarding potential prognosis and severity should be based on the same factors as above. Foremost amongst these is probably the size of the residual D4Z4 fragment (Lunt *et al.*, 1995a). Very broadly, the prediction can be based on the three broad categories given above as types I, II, III (see 21.1) (Lunt *et al.*, 1995b).

Table 21.1 Estimated theoretical distribution of fragment size and type [*Bln*I-resistant (EB) or *Bln*I sensitive (E)] detected on standard gel electrophoresis in FSHD patients and in controls, according to the possible ratios of 4-type : 10-type repeat arrays detected by *Bln*I/*Bgl*II dosage testing or by pulsed-field gel study (PFGE). From this a Likelihood Ratio (LR) of FSHD:control has been calculated for each fragment size/type and each possible EB:E ratio. (Lunt, 2000) (Reproduced with permission from Karger, Basel)

Distribution of fragments in FSHD and controls according to ratio of 4-type (EB): 10-type (E) fragments on PFGE	Conventional gel fragment seen					
	*Bln*I-resistant		No *Bln*I-resistant, but *Bln*I-sensitive….			
	<32kb	32–38kb	<32kb	32–38kb	no frag. <38kb	Total
In FSHD overall	**85%**	**9%**	**4.7%**	**9%**	**0.4%**	**100%**
….with 2EB:2E	73%	8%	0.5%	0.05%	Not FSHD	81 .5%
….with 1EB:2-3E	4%	0.4%	4%	0.8%	0.34%	9.5%
….with 0EB:3-4E	–	–	0.2%	0.05%	0.02%	0.3%
….with 2-3EB:1E	8%	0.9%	0.015%	0.02%	0.04%	9%
….with 3-4EB:0E	0.2%	0.02%	–	–	0.001%	0.2%
In controls overall	**1.5%**	**2%**	**19%**	**39%**	**39%**	**100%**
….with 2EB:2E	0.6%	0.2%	16%	33%	31%	81%
….with 1EB:2-3E	0.01%	0.005%	2%	3.7%	3.8%	9.5%
….with 0EB:3-4E	–	–	0.05%	0.1%	0.1%	0.25%
….with 2-3EB:1E	0.85%	1.7%	1%	1 .8%	3.8%	9%
….with 3-4EB:0E	0.04%	0.1%	–	–	0.01%	0.15%
Likelihood ratios (LR) of FSHD: control						
….with 2EB:2E	120:1	40:1	1:30	1:600	Not FSHD	
….with 1EB:2-3E	400:1	80:1	2:1	1:5	1:10	
….with 0EB:3-4E	–	–	4:1	1:2	1:5	
….with 2-3EB:1E	10:1	1:2	1:60	1:100	1:100	

….with 3-4EB:0E	5:1	1:5	–	–	1:10

Fragment size 7–18 kb (mostly type I)

Severe presentation. Onset expected in childhood. Eventual requirement for a wheelchair expected; mostly by age 40 years, but often by age 25 years.

Fragment size 18–30 kb (mostly type II)

Typical classic presentation. Fifty per cent have clinical signs by 11 years, 95% show signs by age 20 years. Around 10–20% will eventually require wheelchairs. However, quite wide clinical variability can be anticipated, including asymptomatic non-penetrance particularly towards the upper end of fragment size and in females.

Fragment size 30–38 kb (mostly type III)

Presentation will usually be relatively mild, and may involve predominantly shoulder girdle with little or no facial involvement, but there are exceptions. Indeed the inverse corrrelation between severity and fragment size may hold less well with fragment sizes above 30 kb (Butz *et al.*, 2003). The possibility of non-penetrance (i.e. someone remaining asymptomatic) may be quite high, particularly in women heterozygotes (Zatz *et al.*, 1998; Tonini *et al.*, 2003).

However, the possibility of clinical anticipation should also be kept in mind (Lunt *et al.*, 1995b; Zatz *et al.*, 1995; Tawil *et al.*, 1996).

21.3.3 Mild presentation with or without a known family history

Person themselves affected, or clinically asymptomatic but relative affected

When someone shows a relatively mild presentation for FSHD, the genetic advice with respect to offspring and other family members will depend on:

1. whether they could be a somatic mosaic;
2. the fragment size;
3. their sex.

If the consultand appears to be the first case of FSHD in the family, not only should their DNA be checked, but it is highly recommended that DNA samples from both parents should also be studied. If the consultand proves to be a new mutation for a typical D4Z4 deletion, it is quite possible that this could be in mosaic form, since probably at least 40% of new mutations in FSHD occur at somatic mitotic cell division (Upadhyaya *et al.*, 1995; van der Maarel *et al.*, 2000). It may then be possible to prove this from pulsed-field gel analysis, if it can be shown that DNA of the consultand produces five bands recognized by p13E-11 rather than the four expected. Somatic mosaicism will tend to give a milder presentation than a constitutional mutation to the same D4Z4 residual fragment size. In

that situation, affected offspring can be expected to have a more severe presentation than their parent as the consultand. The severity in the offspring will be as appropriate for the particular fragment size present constitutionally. Genetic advice for a female with a mild presentation should certainly indicate a likelihood that affected offspring of hers, particularly if male, may well be more severely affected.

Where someone has a mild presentation in association with a relatively large residual D4Z4 fragment size (30–38 kb), it is most unlikely that this will have arisen from a new mutation in them or even in a recent ancestral generation. Rather, there is likely to be a very extensive family history of other relatives who, if tested, would be shown to harbour the same 'mutation'. The symptomatic person with a fragment >30 kb may merely represent the 'tip of the iceberg' in relation to the number of living or previously deceased family members who carry the mutation. Wider family involvement and testing should therefore be considered, although if the clinical penetrance may be relatively low, the advisability of encouraging wide family testing is less certain. This should in any case only be performed on a voluntary basis, and with full explanation of the limitations of interpretation from the result.

For testing someone who is asymptomatic, but where a relative is known to have a mild presentation, it is imperative that a DNA sample must be obtained and first tested from the relative before offering any predictive test to the consultand. Only if the relative has a typical *Bln*-resistant fragment, and which can be shown to be associated with a 4q35 (type A) telomeric polymorphism can testing reliably be contemplated for the proband, particularly if the anticipated fragment size might be >32 kb. Otherwise a *Bln*-resistant fragment of >35 kb might be coincidental to the FSHD-causing D4Z4 deletion, since *Bln*-resistant fragments of 32–36 kb have now been reported in around 3% of unaffected Caucasian controls (Lunt, 1998; Butz *et al.*, 2003), and from further personal observation, with fragment size of 31–40 kb in 6% of controls.

21.3.4 Severe case in a relative (i.e. in a sib, or in other relative), or parents of a severely affected child (i.e. with same set of parents, or with new partner) or severe case in consultand

Any severe early-onset presentation will almost certainly be associated with a small D4Z4 residual fragment. If it is not, but the clinical diagnosis of FSHD is not in doubt, further DNA tests to check for a deletion encompassing the p13E-11 probe hybridization site must be performed (e.g. by *Bln*I/*Bgl*II dosage test, or telomeric A probe analysis) (Lemmers *et al.*, 1998, 2003). With the exception of the parents or grandparents of a severe early-onset case, one of whom may be mosaic for the D4Z4 deletion, any other relative in the family found to have the same deletion would be expected to show a similar early-onset severe presentation. Therefore, apart from one of the parents, the only other relatives of an apparently sporadic index case who could still be at significant risk for being affected, would be sibs or offspring of the index case. Second-degree and more distant relatives can therefore generally be reassured, even before molecular testing. In practice, the sib of a severe early-onset case can also be offered considerable reassurance if they show no clinical signs by mid-teenage years, and therefore certainly by the time that they themselves might be considering starting a family.

Molecular testing of a relative in the absence of DNA from the index case is helpful in excluding FSHD if no small fragment is seen and they are asymptomatic, or in confirming FSHD if they have suggestive clinical signs and a small *Bln*I-resistant fragment, but could potentially increase uncertainty if a large *Bln*I-resistant fragment (36–45 kb) or a small *Bln*I-sensitive one (<28 kb) is present, as both these can potentially be associated with FSHD (van Deutekom *et al.,* 1996; Butz *et al.,* 2003). In practice, if the affected index case is known to have had early childhood onset and a severe presentation, it is the lack of clinical signs in the asymptomatic relative which provides most information, while the DNA test acts merely to confirm this if it suggests that they are clear, or to support them being clear if it shows a pattern which is anything other than would normally be considered appropriate for a severe early-onset case.

However, this does beg the question of whether there could possibly be genetic susceptibility to new mutation, and therefore whether any unaffected relatives of a severe early-onset new mutation case might also have an increased risk for new mutation in their offspring. At least one pedigree encountered personally, where it seems likely that second cousins have been severely affected, but not intervening relatives, suggests this as a possible consideration, but complete analysis in those families has not been possible, leaving it as a tantalisingly unresolved question. It is believed that the presence of exchange or gene conversion events between chromosome 10-type and 4-type D4Z4 repeats may predispose to *de novo* deletion, since a higher than expected proportion of mosaic cases have *Bln*I-resistant (4-type) repeats on one of the chromosomes 10 (van der Maarel *et al.,* 2000). However, whether the risk of new mutation in offspring of someone with 4-type repeats on chromosome 10, even from a family where there has been a new mutation in a relative, is sufficient to warrant prenatal screening, is another question.

For the offspring of a severe early-onset case, particularly if this is associated with one of the smaller D4Z4 residual fragment lengths (i.e. <17 kb), a similar early-onset severe presentation can be expected if they inherit the affected copy of 4q35. Indeed, the presentation may tend to be more severe if the index case is female and the offspring, a male.

For the parents of a child with a severe early-onset presentation, there is a significant possibility (up to 40% chance) that one of the parents could be a somatic mosaic for the mutation (van der Maarel *et al.,* 2000). This should be checked for as discussed above, since if they are, the risk to further offspring could be as high as 50%. Even if no mosaicism is detected, the possibility of one parent being a germinal mosaic for the mutation would remain and a recurrence risk figure of around 2–3% (1/50–1/30) is probably appropriate (Kohler *et al.,* 1996). The option of prenatal testing by chorionic villus biopsy should be discussed.

Clinical presentation in infantile-onset FSHD

It is in the most severe, early-onset cases of FSHD where pleiotropic effects of the mutation may be important. Not only can there be severe facial weakness resulting in a lack of facial expression (which may lead to initial misdiagnosis of Moebius syndrome) (Padberg, 1982; Jardine *et al.,* 1994b), and early proximal and distal upper and lower limb involvement, but sensorineural hearing loss (Brouwer *et al.,* 1991), visual problems consequent on Coats' disease-like retinal vascular telangiectasis (Fitzsimons *et al.,* 1987),

tongue atrophy (Yamanaka *et al.*, 2001), epilepsy and learning problems can all be associated (Funakoshi *et al.*, 1998). Impaired communication resulting from a combination of lack of facial expression, hearing loss and dysarthria should not be equated with learning difficulty (Gurwin *et al.*, 1985), although this does seem to be a genuine associated problem in the most severe cases. The family described by Small is still perhaps a prime example of infantileonset FSHD (Small, 1968).

For clinical management, it is in the most severe cases where a fluorescein retinal angiogram, regular audiology and retinal screening, and developmental monitoring should be considered.

Whether these additional features represent the influence of additional genes that only become manifest when just one or two D4Z4 repeats remain, or whether they represent an increased chance of effect on the same genes but in a wider range of tissues is still speculative.

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22.
Sarcolemmal reorganization in FSHD

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22.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is linked to deletions of 3.3 kb repeats near the telomeric region of chromosome 4q (Wijmenga *et al.,* 1990, 1991, 1992a,b; Gilbert *et al.,* 1992; Mathews *et al.,* 1992; Mills *et al.,* 1992; Sarfarazi *et al.,* 1992; Upadhyaya *et al.,* 1992; Weiffenbach *et al.,* 1992) which in turn alter the regulation of nearby genes (Gabellini *et al.,* 2002) and possibly other genes as well (Tupler *et al.,* 1999). The mechanism by which these changes become manifest as a muscular dystrophy is still far from clear.

Several muscular dystrophies have been linked to mutations in proteins of the sarcolemma, defined as the plasma membrane of striated muscle, together with closely apposed intracellular and extracellular structures (for recent reviews, see Spence *et al.,* 2002; Michele and Campbell, 2003). Duchenne and Becker muscular dystrophies are linked to mutations in the gene encoding dystrophin (Hoffman *et al.,* 1987, 1988), a major structural protein lining the intracellular surface of the sarcolemma (Koenig *et al.,* 1988; Zubrzycka-Gaarn *et al.,* 1988). Mutations that affect dystroglycan and sarcoglycans, integral membrane proteins of the sarcolemma that associate with dystrophin to form the dystrophin-glycoprotein complex, are linked to muscle-eye-brain disease and Fukuyama muscular dystrophy, and to various limb girdle muscular dystrophies, respectively (Spence *et al.,* 2002; Michele and Campbell, 2003). Similarly, congenital muscular dystrophy with merosin deficiency has been linked to mutations in laminin (Tome *et al.,* 1994; Helbling-Leclerc *et al.,* 1995), a major protein of the basal lamina that lines the extracellular surface of the sarcolemma. Although it has not been shown directly, the changes that occur in these hereditary dystrophies are believed to render the sarcolemma less stable to the stresses exerted during the contractile cycle.

Although the evidence is limited, some studies have shown that the sarcolemma may be affected in FSHD (Schotland *et al.,* 1981; Spuler and Engel, 1998). We have

hypothesized that, as in other muscular dystrophies, FSHD also involves the sarcolemma and, in the absence of direct evidence for changes in the expression of genes encoding sarcolemmal proteins, have devised new approaches to investigating the organization of the sarcolemma to address our hypothesis. Here we show that the organization of the sarcolemma and its relationship to the underlying contractile apparatus is altered in FSHD. We conclude with a model of the sarcolemma and its relationship to the contractile apparatus that can account for our results, and for the weakness and ultimate loss of muscle fibres seen in FSHD.

22.2 Sarcolemmal alterations in FSHD

We applied classic ultrastructural and immunofluorescence approaches to study the sarcolemma in FSHD muscle and in appropriate control samples. Details of our methods will be presented elsewhere. In brief, muscle biopsies (biceps) were collected from FSHD patients whose mutations at 4q35–4q35ter were confirmed by molecular genetic methods. Control specimens consisted of histologically normal muscle biopsies (biceps) collected from patients undergoing evaluation for cramps and myalgias. Samples destined for electron microscopy were clamped close to resting length, fixed in glutaraldehyde, and processed following standard procedures to visualize the sarcolemma and nearby contractile structures. Longitudinal sections were selected for all the analyses presented below. Biopsy samples destined for immunofluorescence were snap frozen at resting length and sectioned longitudinally on a cryostat. The resulting 20 μm thick sections were lifted off the cryostat blade with a fine brush and placed on droplets of phosphate-buffered saline containing 15% ethanol and 10 mM EDTA, to prevent contraction as the frozen sections thawed. Subsequent immunolabelling with antibodies to β-spectrin (Ursitti *et al.*, 2001), a typical membrane cytoskeletal protein that we have often used to study sarcolemmal organization, followed our established procedures for indirect immunofluorescence (Williams and Bloch, 1999a).

Results from both ultrastructural and immunofluorescence studies indicate that the sarcolemma is significantly modified in FSHD.

Indirect immunofluorescence labelling of tangential, longitudinal cryosections of the sarcolemma typically shows regular, linear arrays enriched in β-spectrin. In control samples *(Figure22.1A)*, these sarcolemmal structures, termed ‘costameres’ (Craig and Pardo, 1983; Pardo *et al.*, 1983; Street, 1983; Porter *et al.*, 1992), are oriented transversely and overlie the Z-lines (arrow) and M-lines (arrowhead) of the nearby contractile apparatus. Specifically, the thicker transverse structures overlie the Z-lines, and the thinner structures overlie the M-lines of nearby myofibrils (Porter *et al.*, 1992; Williams and Bloch, 1999b; O’Neill *et al.*, 2002). Their consistent dimensions and location in the sarcolemma suggest that the processing of these samples in the cryostat, and their subsequent labelling with antibodies, does not significantly disrupt the organization of the sarcolemma, which in most respects resembles images we have obtained from rodent muscle that had been fixed before cryosectioning (Williams and Bloch, 1999a,b; O’Neill *et al.*, 2002). The one significant difference we have noted between control human and rodent samples is the absence of longitudinally oriented costameric structures in the human samples. As their presence in rodent muscle is

variable, however, we do not ascribe much importance to their absence in human biopsy samples.

When we subjected snap frozen samples of biceps muscles from FSHD patients to identical procedures, we obtained results that were significantly different *(Figure 22.1B)*. Although labelling for β-spectrin in the Z-line domains (arrow) was usually similar to that of controls, distinct M-line domains were difficult to detect. In their place, we usually found structures that, rather than being oriented parallel to Z-line domains and transverse to the fibre axis, were oriented at a diagonal (*Figure 22.1B*, long arrows). We have examined muscle samples from several different human and rodent muscular dystrophies and myopathies but have never seen structures like these in samples other than FSHD biopsies. As these diagonal structures were commonly observed in FSHD but not in other dystrophic or myopathic samples, we believe that they may be diagnostic for the disease. In any case, their presence clearly indicates that the organization of the sarcolemma is altered in muscle from FSHD patients. In particular, our results show that the sarcolemmal membrane skeleton at Z-line domains but not M-line domains remains aligned with the underlying contractile apparatus.

We also used ultrastructural techniques to study the sarcolemma and its relationship to contractile elements. Electron microscopy of thin sections, cut parallel to the long axis of the biopsied myofibres, reveals the sarcolemma, including the basal lamina and extracellular matrix, as well as the nearby contractile apparatus

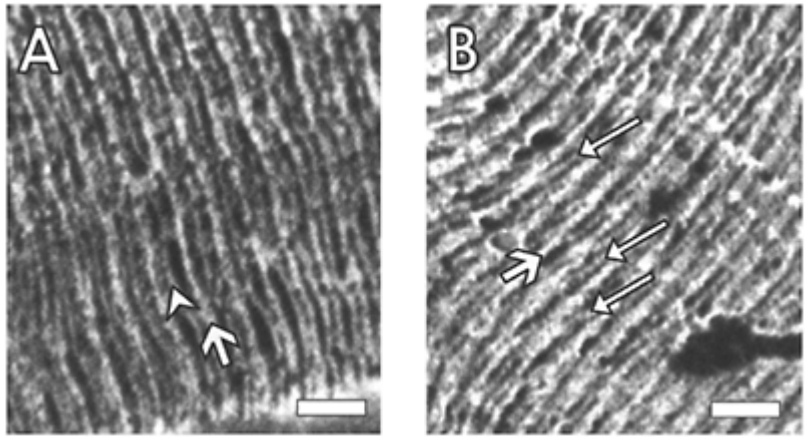

Figure 22.1 Altered distribution of β-spectrin in costameres of FSHD muscle. Snap frozen samples of biceps muscle from control and FSHD patients were sectioned on a cryostat in the longitudinal direction, collected on slides under conditions that minimized contraction (see Methods), and then

labelled by indirect immunofluorescence with antibodies to-spectrin, a marker for costameres. Controls (A) showed normal costameric labelling of thick sarcolemmal domains lying over the Z-lines (arrow) and thinner sarcolemmal domains overlying the M-lines (arrowhead) of nearby myofibrils. FSHD samples, by contrast, showed the thicker domains overlying Z-lines (arrow), but structures overlying M-lines were hard to detect. Instead, they were often replaced by structures that coursed diagonally between neighbouring Z-line domains (long arrows). The results show that the membrane skeleton at the sarcolemma of FSHD muscle is altered. Bars, 5 μm.

(Figure 22.2). Typically, in control muscle fibres, the sarcolemma was tightly and nearly uniformly apposed to the nearby contractile apparatus *(Figure 22.2A)*. Occasionally, lipid droplets or mitochondria were interposed between the contractile apparatus and the sarcolemma, but when this occurred, the sarcolemma closely adhered to the shape of the underlying organelle and immediately adjacent to these structures was again in close apposition to the contractile apparatus (not shown). By contrast, the sarcolemma in most of the regions we studied in electron micrographs of FSHD muscle was more distant from the underlying myofibrils *(Figure 22.2B)*. When lipid droplets or mitochondria were interposed, the sarcolemma did not follow their shapes, nor did it become more closely associated with the contractile apparatus at adjacent sites, suggesting that intracellular organelles were not responsible for the displacement of the plasma membrane from the underlying myofibrils. Instead, the sarcolemma was separated by a more or less constant gap from the myofibrils over distances of several sarcomere lengths.

We quantitated our observations by measuring the distances between the sarcolemma and the Z-lines of the underlying myofibrils in 62 regions of myofibres from four samples from three control patients and in 32 regions of myofibres from four samples from four patients with FSHD *(Figure 22.3)*. To minimize the effects caused by the presence of subsarcolemmal organelles, we limited our measurements to regions where there were no large organelles that could displace the membrane near the sites we analyzed. Measurements in control muscles gave values of 46.7±29.4 nm (mean±S.D.), whereas FSHD samples gave values of 222±124 nm. These differences were highly significant ($P<0.001$, Mann-Whitney test). Although we have not yet found other structural

differences between the sarcolemmae of control and FSHD muscles, these results, like the results of our

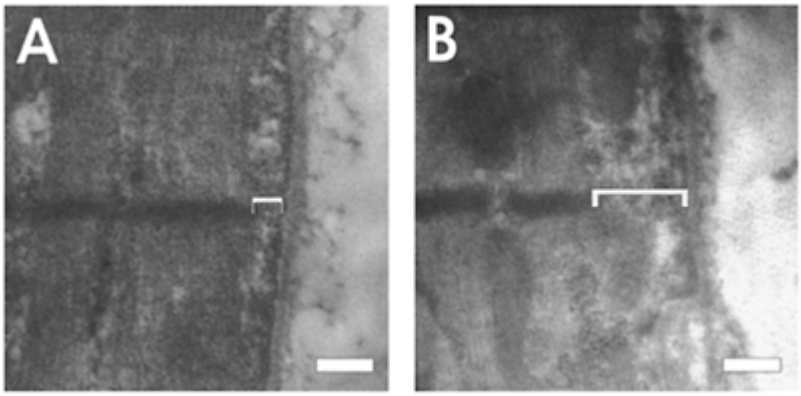

Figure 22.2 Ultrastructural changes near the sarcolemma of FSHD muscle. Samples of biceps muscle from control and FSHD patients were processed for thin-section electron microscopy following standard techniques, and sectioned longitudinally. Fibres were visualized at the level of the sarcolemma at high magnification. Control myofibres routinely showed only a small gap between the sarcolemma and the ends of the Z-lines of the most superficial myofibrils (A, bracket). FSHD samples routinely showed larger gaps (B, bracket). The results suggest that the links of the sarcolemma to the underlying contractile apparatus are weakened in FSHD. Bars, 250 nm.

immunofluorescence studies, indicate that the relationship of the sarcolemma to the underlying contractile apparatus is altered in FSHD.

22.3 Sarcolemmal involvement in FSHD and other dystrophies

Muscular dystrophies linked to dystrophin and proteins of the dystrophin-associated glycoprotein complex, as well as to laminin, have been hypothesized to be caused by a weakening of the links between the contractile apparatus and the basal lamina of affected skeletal muscle fibres (reviewed in Spence *et al.*, 2002; Michele and Campbell, 2003). We have found that dystrophinopathies in mice and humans also result in changes in the organization of the sarcolemma, such that the usual alignment of costameres with the underlying contractile apparatus is disrupted (Porter *et al.*, 1992; Williams and Bloch, 1999b; our unpublished results). We used this observation as a point of departure to study FSHD, and, in particular, to address the question, 'Is the organization of the sarcolemma and its relationship to the underlying contractile apparatus altered in FSHD?'. Our present results answer this question in the affirmative: the organization of the sarcolemma in FSHD is indeed altered. Furthermore, the alterations may be distinctive to FSHD and may account for the muscle weakness and fibre loss seen in this disease.

Our immunofluorescence studies indicate that the sarcolemma of FSHD muscle becomes reorganized from a structure that contains transverse domains overlying Z-lines and M-lines to a structure with transverse domains limited to Z-lines and diagonal structures *(Figure 22.1)*. We have been unable to detect any changes in the organization of the contractile apparatus in the vicinity of these diagonal structures, suggesting that the co-alignment typical of the sarcolemma of control

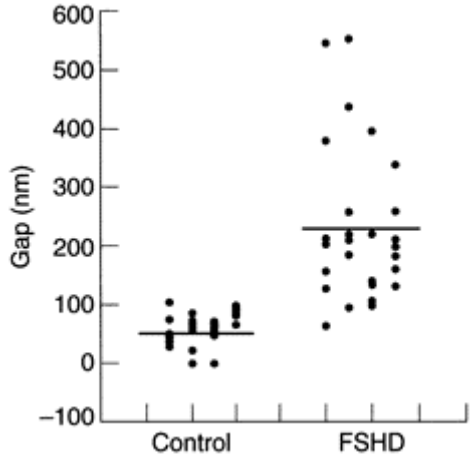

Figure 22.3 Quantitation of gaps between the sarcolemma and the contractile apparatus. Electron micrographs were analysed by measuring the distances indicated by

the brackets in Figure 22.2. Four samples from three control patients and four samples from four different FSHD patients were analysed. The controls all clustered around a mean value of ~50 nm (bar). FSHD samples showed more variability, but showed a mean of ~220 nm (bar). The differences were highly significant ($P<0.001$). These results confirm that the links between the sarcolemma and the underlying contractile apparatus are altered in FSHD muscle.

muscle has been disturbed in FSHD. Neither we, nor others, have found diagonal structures in other human or mouse muscular dystrophies. As control muscle biopsies processed identically do not exhibit diagonally oriented structures at the sarcolemma, their appearance in FSHD samples are not simply an artifact of the procedures used to section and label the (unfixed) muscle samples we study. Thus, the presence of diagonal β-spectrin-rich structures at the sarcolemma may be a reliable, and perhaps even distinctive, feature of FSHD muscle. Our experience in processing unfixed human muscle samples from dystrophic or myopathic patients is still limited, however. Further studies will be required to determine if these structures are truly diagnostic of FSHD and, if so, if their prevalence or morphological properties vary with the severity of the disease, or, alternatively, if they are an architectural feature of the sarcolemma that FSHD shares with other diseases of muscle.

Our ultrastructural and immunofluorescence studies both indicate that the relationship of the sarcolemma to the underlying contractile apparatus is altered in FSHD. The paucity of costameric elements aligned with nearby M-lines is indicative of this, as is the increased separation between the sarcolemma and Z-lines of the nearby contractile apparatus that we measured in FSHD muscle *(Figures 22.2, 22.3)*. For quantitations, we performed our measurements at sarcolemmal sites overlying Z-lines, and not M-lines, both because Z-lines are more reliably identified in longitudinal sections, and because the structures that replace the M-line domains at the sarcolemma of FSHD muscle are clearly abnormal. The 4–5-fold increase in the distance from the sarcolemma to the perimeter of the Z-line was striking. Indeed, few (~16%) of the FSHD fibres yielded values in the control range, and all four FSHD samples yielded comparable results *(Figure 22.3)*. Nevertheless, the fact that the Z-line domains of costameres remain aligned with the Z-lines of nearby myofibrils strongly suggests that the sarcolemma is still connected to the contractile apparatus at these sites.

22.4 Conclusion and model

Links between the myofibrils and the sarcolemma are required to transmit some of the force of contraction to the extracellular matrix, and thence to the tendon (Street, 1983; reviewed in Bloch and Gonzalez, 2003). The loss of these links, which occurs in diseases such as Duchenne muscular dystrophy and muscle-eye-brain disease, in which particular proteins forming the links are absent or altered (Spence *et al.*, 2002; Michele and Campbell, 2003), can result in muscle weakness and the loss of muscle fibres. Muscle weakness in these and related diseases may be due to the fact that, although dystrophic muscle continues to generate nearly normal levels of contractile force, the dystrophic sarcolemma is able to transmit less force than the control sarcolemma. Fibre loss may be caused by a mismatch between the normal contractile forces that are generated and an unstable sarcolemma, compromised by the absence or alteration of key proteins.

Our results show that the organization of the sarcolemma of FSHD muscle is altered and that the closeness of its links to the underlying contractile apparatus is compromised. The latter alone may be sufficient to account for muscle weakness in FSHD. As illustrated in *Figure 22.4A,B*, the tight coupling of the sarcolemma to the contractile apparatus of control muscle can result in efficient translation of the movement generated by actomyosin interactions to the sarcolemma and its associated matrix. In FSHD muscle, by contrast, because of its increased distance to the sarcolemma, the contractile apparatus in each sarcomere is likely to have to undergo greater activity before the force of contraction is transmitted to the sarcolemma to cause its displacement *(Figure 22.4C,D)*. Thus, the increased size of the gap between the superficial myofibrils and the sarcolemma is likely to have physiological consequences for muscle strength.

Our results also suggest a possible mechanism of fibre loss in FSHD. We hypothesize that the increased gap between the sarcolemma and the superficial myofibrils leads to the generation of shear forces under the sarcolemma that

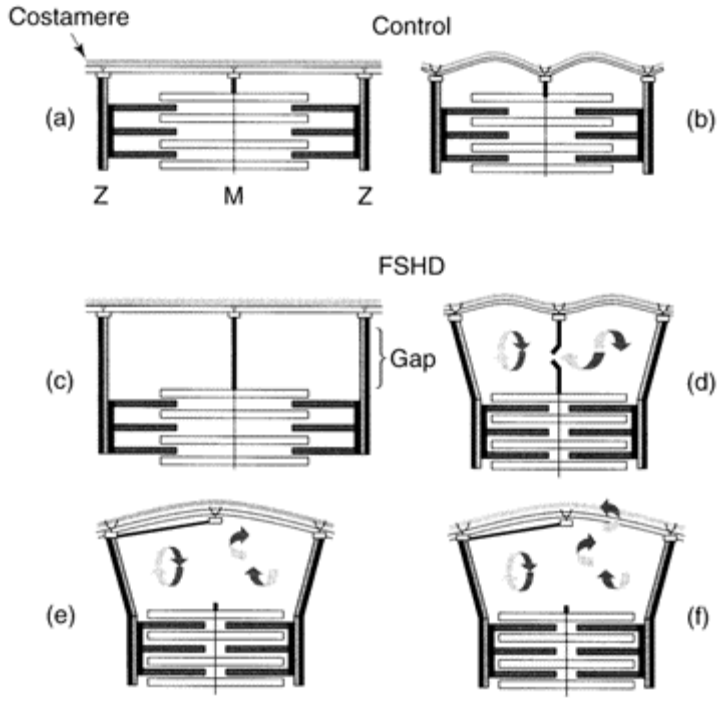

Figure 22.4 Model. Although aspects of this model are supported by our results, key features must still be addressed experimentally. (A) In control muscle fibres, the gap between the sarcolemma and the nearby myofibrils is small, and connections are maintained at costameres that exist at the levels of the Z- and M-lines. (B) As control muscle contracts, the close links between the sarcolemma and the superficial myofibrils ensure that the movement of the sarcolemma is closely coupled to that of the sarcomeres, thereby transmitting some of the force of contraction across the membrane to the extracellular matrix. (C) In FSHD muscle fibres, the gap between the sarcolemma and the

nearby myofibrils is large, and although connections exist at the level of the Z- and M-lines (as shown here), the structures lying over M-lines are susceptible to reorganization (see below). (D) The larger sarcolemma-to-myofibril gap reduces the efficiency with which contractile force is transmitted across the cell membrane to the matrix, thereby causing muscular weakness. Damage to the costameric domains overlying M-lines is hypothesized to occur as a result of shear forces (arrows) made possible by the movement of cytoplasmic material between the myofibrils and the sarcolemma. (E) Breakage of the connections at M-line domains in FSHD may permit them to form diagonally oriented structures. (F) Further shear-induced damage is hypothesized to rupture the plasma membrane, ultimately causing loss of the myofibres.

break the links between M-lines and the M-line domains of costameres *(Figure 22.4D)*. We speculate further that these breaks cause both a reorientation of the structures responsible for these links, to form diagonal elements at the sarcolemma *(Figure 22.4E)*, and an increased susceptibility of the sarcolemma to damage due to shear forces generated during the contractile cycle *(Figure 22.4F)*. *In vitro* studies of the sarcolemma of FSHD muscle will be required to test these ideas critically, and to generate new hypotheses, should these prove to be wrong.

22.5 Future directions

Although our results indicate that the organization of the sarcolemma of FSHD muscle, and its structural relationship to the underlying contractile apparatus, are altered in ways that may compromise its contractile strength, and even its survival, further analysis will be required to determine if the changes we have documented are diagnostic for FSHD or if they have some other explanation. Several other possibilities need to be addressed. For

example, partial shifts in fibre type, which may occur in FSHD (Tupler *et al.,* 1999), could account for some of our observations. Some of the changes we have observed may also be shared by other myopathies or dystrophies that affect the sarcolemma. More extensive immunofluorescence studies of the costameric organization of the sarcolemma in human muscle, and how it is altered in different muscle diseases, are sorely needed. Similarly, quantitative ultrastructural studies of muscle from patients diagnosed with other muscular dystrophies or myopathies are needed to determine if the distances between the sarcolemma and myofibrils really are selectively increased in FSHD, or if this is a feature common to other dystrophies. Finally, molecular and proteomic dissection of the membrane-associated complexes at the costameres that are altered in FSHD, and their possible linkage to the changes in gene expression associated with deletions at 4q35–4q35ter, may provide more definitive evidence for changes that occur at the sarcolemma of FSHD muscle.

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23.
Expression profiling in FSHD

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23.1 Introduction

23.1.1 Deletion of D4Z4 in FSHD

FSHD is an autosomal dominant neuromuscular disease that initially affects the facial, shoulder girdle and upper arm musculature (Tawil *et al.,* 1998; Padberg and Adams, 2000). Asymmetric involvement of specific muscle groups is common and occasionally the complete absence of a muscle, e.g. the pectoralis, is noted. Such findings suggest a developmental defect, although FSHD is often not recognized clinically until the second or third decade of life (Lunt *et al.,* 1989). Extramuscular manifestations of the disease frequently include retinal vascular anomalies, sensorineural hearing loss and, in severe cases, epilepsy and mental retardation (Padberg *et al.,* 1992; Funakoshi *et al.,* 1998). Males are often more severely affected and have an earlier age of onset than females (Zatz *et al.,* 1998).

The genetic mutation responsible for FSHD involves a deletion of D4Z4 repeats (van Deutekom *et al.,* 1993). D4Z4 is a 3.3 kb subtelomeric tandem repeat on the distal long arm of chromsosome 4. FSHD is associated with an *Eco*RI fragment that contains fewer than 11 copies of the D4Z4 repeat (Wijmenga *et al.,* 1992). There is a general inverse correlation between the size of this fragment and the severity of the disease (Lunt *el al,* 1995; Tawil *et al.,* 1996). Homologous D4Z4-like repeats are also present in the subtelomeric region of chromosome 10q. Since the sequence is not identical, the 4q repeat block can be identified by *Eco*RI/*Bln*I digestion (Deidda *et al.,* 1996). Exchange of homologous D4Z4-like repeats between chromosome 10qter and 4qter results in FSHD, if the resulting number of these repeats is fewer than 11 on chromosome 4 (Lemmers *et al.,* 1998). Thus, it is the absolute number of D4Z4 repeats at 4qter and not their precise sequence that is critical for disease manifestation.

23.1.2 Chromatin structure of D4Z4

Several lines of evidence suggest that D4Z4 is transcriptionally inert and/or embedded in heterochromatin, the highly compacted, often transcriptionally silent complex of DNA and protein *(Box 23.1)*. The D4Z4 repeat has several characteristics of heterochromatin, the highly condensed chromosomal structure often responsible for gene silencing (Richards and Elgin, 2002). D4Z4 exists as a tandem repeat sequence which lies immediately adjacent to the telomere of human chromosome 4q and has significant sequence similarity to known classes of constitutive heterochromatin (Bengtsson *et al.*, 1994; Hewitt *et al.*, 1994; Winokur *et al.*, 1994). D4Z4 cross-hybridizes to homologous loci in the human genome, predominantly in regions known to be heterochromatic (Winokur *et al.*, 1994; Lyle *et al.*, 1995). In addition, chromatin structure analysis reveals that the D4Z4 repeat is associated with the same histone H1 subtype as telomeric heterochromatin (Parseghian *et al.*, 2000). The D4Z4 repeat on normal chromosomes is highly methylated and contains hypoacetylated histones, common characteristics of transcriptionally silent chromatin (Tsien *et al.*, 2000; Jiang *et al.*, 2003; Chapter 17). The distal 4q chromosomal region is extremely gene-poor. A homeodomain (DUX4) is found with an open reading frame in D4Z4 (Gabriels *et al.*, 1999), but to date there is no compelling evidence for expression of this putative gene *in vivo*. However, the possibility that this gene may be aberrantly transcribed in a cell- or developmental-specific fashion in FSHD cannot be excluded.

23.1.3 Regulation of gene expression

Recent data lend strong support to the consideration of FSHD as a disease of chromatin. A polymorphism of the 4q telomere exists in the population with nearly equal frequencies (van Geel *et al.*, 2002). FSHD is uniquely associated with the 4qA allele variant containing β-satellite, a sequence previously associated with heterochromatin (Lemmers *et al.*, 2002). In addition, many D4Z4 CpG methylation-sensitive restriction sites are significantly hypomethylated in FSHD patients as compared to control individuals (van Overveld *et al.*, 2003). Strikingly, this hypomethylation is also seen in FSHD patients with no linkage to 4q and with no contraction of D4Z4 repeats, thereby strengthening the argument that D4Z4 hypomethylation plays an important role in the pathogenesis of FSHD. A multi-

1 Exists as a tandem, repetitive sequence block
2 Maps adjacent to the telomere (subtelomeric region)
3 Sequence similarity to Lsau, hhspm3 heterochromatin
4 Dispersion to heterochromatic regions (acrocentrics, 1q12)
5 Same histone H1 subtype as telomeric heterochromatin
6 Large blocks of D4Z4 are highly methylated, hypoacetylated
7 In close proximity to β-satellite heterochromatic repeat
8 No evidence of DUX4 expression *in vivo*

Box 23.1 D4Z4: Heterochromatin or silent euchromatin

protein complex that binds to D4Z4 *in vivo* is proposed to negatively regulate gene expression (Gabellini *et al.*, 2002), with D4Z4 deletions in FSHD permitting increased gene transcription at 4q35. These differences in chromatin structure of the FSHD region may affect the regulation of genes on 4q35 or, by virtue of altered nuclear localization and sequestration of chromatin proteins, elsewhere in the genome. This review of expression profiling in FSHD describes current insights into the genes and cellular pathways affected in FSHD muscle. Initial focus on these genes was gained from global gene expression studies and further examination of their role was carried through cell culture experiments. A discussion of recent data on D4Z4 nuclear positioning is included, as it serves to integrate many aspects of FSHD pathogenesis.

23.2 Genome-wide expression profiling

23.2.1 Overview

As FSHD arises from a complex regulatory mutation, methods to examine global gene expression are well suited to decipher the molecular pathology of this disease. Examination of gene expression at 4q35 will probably identify the primary genes responsible for the disease, while examination of genome-wide gene expression should provide greater insight into the molecular and cellular pathways disrupted in the disease. Previous studies have utilized subtractive hybridization to identify global misregulation of genes involved in FSHD pathology (Tupler *et al.*, 1999). More recently, the development of microarray technology has allowed enhanced sensitivity and selectivity in differential RNA expression levels (Brown and Botstein, 1999; Lipshultz *et al.*, 1999). Microarrays have been used now for several years to study gene expression in such situations as analysis of disease versus normal tissues, profiling tumours and examining gene expression during development. The expression-profiling methodologies currently utilized for FSHD are described below.

23.2.2 Oligonucleotide arrays

Affymetrix GeneChips are *in situ* synthesized microarrays containing hundreds of thousands of oligonucleotide probes at extremely high densities (Lipshultz *et al.*, 1999). The probes are designed to optimize sensitivity, specificity, and reproducibility of relative RNA expression levels. Such oligonucleotide probes allow for a low signal-to-noise ratio, as well as discrimination between the closely related target sequences often found in gene families. Biotin-labelled cRNA generated from FSHD muscle and myoblast cell cultures is hybridized to the array, detected with phycoerythrin-streptavidin and the resultant fluorescent images read with an array scanner. Following analysis of hybridization intensities with GeneChip software, data can be exported as text files for statistical analysis. One such statistical program is Cyber T, which is based on the Student's *t*-test and is designed for output data from large-scale microarray experiments (Baldi and Hatfield, 2002). Regularized *t*-tests between experimental groups are generated within a Bayesian statistical framework. Estimates of variance of expression levels for each gene are therefore improved by including the variance of other genes with

similar expres-sion levels. Transcripts are considered to be significantly dysregulated if they display a twofold or greater fold change and have a *P* value less than or equal to 0.05.

23.2.3 cDNA arrays

cDNA arrays are produced by depositing presynthesized or cloned cDNAs onto glass slides (Brown and Botstein, 1999). Sample and reference RNAs are then labelled with either Cy3 or Cy5, hybridized simultaneously to the array, and the relative fluorescent signals analysed for differential expression levels. cDNA arrays have several major advantages over commercial microarrays including their relatively low cost and the ability to be produced in an academic setting. Thus, investigators can design expression arrays for several hundred to several thousand genes of particular interest to their field of study. cDNA microarrays, however, have a lower sensitivity and specificity for differential RNA detection than oligonucleotide arrays. This occurs in large part because members of related gene families cannot be distinguished when probed with total cellular RNA. This is a concern when studying diseases that map to highly repetitive regions, such as FSHD. The problem is exemplified by two genes, *FRG1* and *FRG2* (Chapter 5), which map to the repetitive region proximal to D4Z4 and are also present at multiple loci elsewhere in the genome.

FRG1 is a highly conserved gene which, although single-copy in pufferfish and mouse, has been greatly amplified and dispersed throughout the genome in the great apes and man (Grewal *et al.,* 1999). Subcellular localization studies identify the nucleoli and Cajal bodies as putative sites of the FRG1 protein, functioning perhaps in RNA processing or transcriptional regulation (Chapter 5). *FRG2* is a muscle-specific transcript present only in primates (Chapter 5). The predicted protein is localized to the nucleus but exhibits no homology to known proteins.

The role of these two genes in FSHD has yet to be elucidated. Several studies indicate that *FRG1* and *FRG2* are up-regulated in FSHD myoblasts and mature muscle, although experiments conducted in various laboratories yield conflicting results (Jiang *et al.,* 2003; Winokur *et al.,* 2003b; Gabellini *et al.,* 2002). Unfortunately, global expression profiling cannot contribute to the analysis of these two genes in FSHD because current microarray platforms cannot distinguish between the FSHD-region copy of these genes and pseudogenes on other chromosomes. However, a custom cDNA array, containing all 4q35 and 10q26 genes and ESTs, has been designed and constructed in order to examine the differential expression of all single copy transcripts in the FSHD region. The results of these experiments are described below.

23.3 Expression profiling of FSHD muscle

23.3.1 Cell cycle and differentiation genes

Gene expression profiling of FSHD muscle suggests an intriguing hypothesis for the pathophysiological basis of FSHD: a primary defect in muscle cell differentiation (Winokur *et al.,* 2003b). Many of the genes dysregulated in FSHD are involved in

myogenesis, cellular differentiation and cell cycle control *(Table 23.1)*. Aberrant transcription of genes involved in differentiation and proliferation could either result from an intrinsic defect in FSHD muscle or from enhanced regeneration and recruitment of satellite cells in affected muscle. However, the genes described here are deregulated in an FSHD disease-specific manner and are thus likely to be involved in an FSHD-specific differentiation defect, rather than to be the result of secondary dystrophic changes. In addition, the genes mis-expressed in FSHD are highly enriched for genes/proteins that interact with, or are direct targets of, MyoD, suggesting an enhancement of an early sub-programme of MyoD-mediated muscle cell differentiation (Winokur *et al.*, 2003b).

FSHD and control muscle biopsy samples were examined by GeneChip oligonucleotide expression arrays. The expression data were compared to those generated for other types of muscular dystrophy, including Duchenne muscular dystrophy (DMD) and α-sarcoglycan deficiency (α-SGD) (Chen *et al.*, 2000). Of

Table 23.1 Cell cycle and differentiation genes altered in FSHD

Gene description	Symbol	Location	Fold change
MyoD family inhibitor	*MDFI*	6p21	33.3
caspase 1, apoptosis-related cysteine protease	*CASP1*	11q23	10.0
wee1+*(S. pombe)* homologue	*WEE1*	11p15.3–p15.1	7.6
transferrin receptor	*TFRC*	3q26.2–qter	7.4
cyclin B1	*CCNB1*	5q12	7
growth arrest-specific 6	*GAS6*	13q34	4.4
caspase 2, apoptosis-related cysteine protease	*CASP2*	7q34–q35	4.3
cardiac LIM protein; cysteine- and glycine-rich protein 3	CLP	11p15	12.9
creatine kinase, brain	*CKB*	14q32	2.7
mitogen-induced nuclear orphan receptor	*KIAA0195*	17	2.7
growth arrest-specific 7	*GAS7*	17p	2.6
mitogen-activated protein kinase-activated protein kinase 2	*MAPKAPK2*	1q32	2.5
delta-like homologue *(Drosophila)*	*DLK1*	14q32	2.4
cyclin D2	*CCND2*	12p13	2.4
cysteine and glycine-rich protein 3 (muscle LIM protein)	*CSRP3*	11p15.1	2.4
cyclin D1	*CCND1*	11q13	2.2
insulin-like growth factor-binding protein 4	*IGFBP4*	17q12–q21.1	2.1

immediate early protein	*ETR101*	19p13.2	–2.1
TGFB-inducible early growth response	*TIEG*	8q22.2	−2.2
transforming growth factor, beta 3	*TGFB3*	14q24	−2.3
cyclin G2	*CCNG2*	4q21.1-q21.3	−2.4
metallothionein 1L	*MT1L*	16q13	−2.6
metallothionein 1E (functional)	*MT1E*	16q13	−2.7
CASP8 and FADD-like apoptosis regulator	*CFLAR*	2q33–q34	−2.7
early growth response 1	*EGR1*	5q31.1	−2.8
insulin-like growth factor binding protein 5	*IGFBP5*	2p21	−3.1
myocyte-specific enhancer factor 2	*MEF2*	6p21.3	−3.3
metallothionein 1H	*MT1H*	16q13	−3.6
cyclin G2	*CCNG2*	4q28	−3.8
immediate early protein	*ETR101*	19	−4.0
metallothionein 2A	*MT2A*	16q13	−5.1

the FSHD-specific dysregulated genes, a large number have a role in cellular proliferation and differentiation. Muscle LIM protein (MLP), also known as cysteine and glycine-rich protein 3 (CSRP3), is increased nearly threefold, whereas this transcript is not altered in the other types of muscular dystrophy studied. MLP functions as a positive regulator of myogenesis (Arber *et al.*, 1994; Kong *et al.*, 1997). Another gene involved in the differentiation of many cell types and up-regulated in FSHD muscle is the *Drosophila* delta-like homologue *DLK1* (Laborda, 2000). The transcription of *WEE1*, a Cdk-inhibitory kinase that functions in part to arrest the cell cycle (Igarashi *et al.*, 1991), is elevated in FSHD. The transferrin receptor *(TFRC)* gene is also up-regulated in FSHD muscle. Transferrin is a key myoblast trophic factor, initially promoting myoblast proliferation, and subsequently supporting myogenic differentiation (Chen and Quinn, 1992). Two other genes, very low density lipoprotein receptor *(VLDLR)* and necdin *(NDN)*, which play important roles in differentiation, are also up-regulated in FSHD muscle. *VLDLR* is required for neurogenesis of the cerebral cortex (Trommsdorff *et al.*, 1999) and *necdin* is an imprinted gene that suppresses growth in postmitotic neurons (Nakada *et al.*, 1998).

Other genes of interest in the light of FSHD pathophysiology include immunoglobulin lambda-like polypeptide 2 *(IGLL2)* and thioredoxin interacting protein *TXNIP* (also known as vitamin D3 up-regulated protein 1, VDUP1). Since FSHD muscle often has marked inflammatory infiltrates (Arahata *et al.*, 1995), identifying specific genes involved such as *IGLL2* may prove valuable in dissecting the role of the immune response in this disease. TXNIP functions as an oxidative stress mediator by inhibiting activity of thioredoxin, a potent thiol reductase and reactive oxygen species regulator (Junn *et al.*, 2000). *TXNIP* is down-regulated in FSHD muscle, a finding consistent with the enhanced vulnerability to oxidative stress seen in FSHD myoblasts (Figlewicz *et al.*,

2002; Winokur *et al.*, 2003a; Chapter 16). Interestingly, a number of metallothionein (MT) transcripts are also reduced in FSHD muscle. Metallothioneins are a group of ubiquitous low-molecular-weight proteins that have functional roles in cell growth, repair and differentiation. MTs serve to protect against free radical toxicity during the differentiation of myoblasts to myotubes (Apostolova *et al.*, 1999).

23.3.2 Common changes in muscular dystrophy

Not surprisingly, a number of genes with altered expression in FSHD are also dysregulated in other forms of muscular dystrophy. Of 131 genes altered in Duchenne and α-sarcoglycan muscular dystrophy (Chen *et al.*, 2000) using the identical GeneChip format, 35 (27%) are also altered in FSHD. Several of the dysregulated genes in FSHD muscle that were also found to be affected in other types of muscular dystrophy (DMD, αSGD) are listed in *Table 23.2*. A large proportion of these genes are probably dysregulated as a result of fibrotic infiltration. These genes include such extracellular matrix proteins as collagen types I and VI, lumican, fibronectin and tenascin. Other categories of genes affected, in common with other types of muscular dystrophy, include immune response genes such as complement and coagulation factors, and genes encoding proteins involved in energy metabolism.

Table 23.2 Gene dysregulated in common with other muscular dystrophies

				Fold change		
NM	Accession No.	Gene		FSHD	DMD	α-SGD
		Cell surface and extracellular matrix				
NM-000088	M55998	collagen type I alpha-1	*COL1A1*	5	6	5
NM-001849	X58251	collagen, type VI, alpha 2	*COL6A2*	2	nc	6
NM-000089	Z74616	collagen, type I, alpha 2	*COLA2*	5	6	5
NM-2026	X02761	fibronectin 1	*FN1*	3	4	3
NM-002345	U21128	lumican	*LUM*	2	5	5
		Energy metabolism				
NM-000237	M15856	lipoprotein lipase	*LPL*	3	−2	−5
NM-003356	AF001787	uncoupling protein 3	*UCP3*	−6	nc	–9
		Muscle structure and development				

NM-005159	J00073	actin, alpha, cardiac muscle	*ACTC*	3	7	9
NM-002470	X13988	myosin, heavy polypeptide 3, skeletal muscle	*MYH3*	3	140	124
		Immune response				
NM-001734	J04080	complement component 1, s subcomponent	*C1S*	2	5	3
NM-002113	M65292	H factor (complement)-like 1	*HFL1*	2	5	3
NM-001733	M14058	complement component 1, r subcomponent	*C1R*	2	5	4
		Intracellular and cell-cell communication				
NM-001552	M62403	insulin-like growth factor-binding protein 4	*IGFBP4*	2	5	4
NM-002966	M38591	S100 calcium-binding protein A10	*S100A10*	2	5	6
NM-018417	AL035122	soluble adenylyl cyclase	*SAC*	−11	−6	−3
		Others and unknown				
NM-000067	Y00339	carbonic anhydrase II	*CA2*	−3	−4	−4
NM-000129	M14539	coagulation factor XIII, A1 polypeptide	*F13A1*	3	26	11
NM-003430	L11672	zinc finger protein 91	*ZNF91*	−3	−5	−6
NM-000156	Z49878	guanidinoacetate N-methyltransferase	*GAMT*	−2	−4	−3
NM-005127	X96719	C-type lectin, superfamily member 2	*CLECSF2*	5	3	4
Nm-005824	U32907	37 kDa leucine-rich repeat (LRR) protein	*LRRC17*	23	12	nc

nc=no change in expression level

23.3.3 Position effect variegation

The likely function of the trancriptionally inert repeat D4Z4 at 4qter is to establish and/or maintain a chromosomal environment in which genes can be expressed in a tissue- or development-specific manner. Changes in chromatin structure occur upon deletion of D4Z4 repeats in FSHD, changing from a classical heterochromatic (transcriptionally silent), highly methylated sequence to one that is more permissive of gene transcription

(Sections 23.1.2 and 23.1.3). FSHD has been postulated to result from aberrant gene transcription mechanistically related to position effect variegation (PEV) in *Drosophila*, the repression of neighbouring gene expression by highly compacted, transciptionally silent heterochromatin (Elgin, 1996). Heterochromatin also mediates transcriptional silencing of telomere-proximal genes and the nearby silent mating type loci (HM) in yeast (Grunstein, 1997). A generalized up-regulation of genes resulting from deletions of the heterochromatic D4Z4 repeat could explain the autosomal dominant pattern of FSHD. As described in Chapters 5 and 10, three genes, *FRG1, FRG2* and *ANT1,* have been proposed to be up-regulated in FSHD. Based upon these results, a position effect is suggested to extend throughout the FSHD gene region at 4q35. Such a region-wide position effect in FSHD can be directly tested for using gene expression profiling of FSHD muscle on oligonucleotide and cDNA microarrays.

23.3.4 4q35 regional gene expression

Oligonucleotide and cDNA microarrays are well suited to detect differential expression of single-copy genes. RNA expression of genes and ESTs localizing to the FSHD region and the homologous region at 10q26 was analysed both by the Affymetrix GeneChip and a custom FSHD array (Winokur *et al.,* 2003b). This custom cDNA microarray consists of 76 genes and ESTs from chromosome regions 4q35 and 10q26. The vast majority of these genes are single-copy, unique sequences and thus can be used to test the position effect model in FSHD. As mentioned above, a number of multi-copy genes such as *FRG1* and *FRG2* cannot be analysed using array methodologies. As expected, we find no elevation of *FRG1* or *FRG2* upon microarray analysis of FSHD muscle, probably because we are not able to discern 4q35-specific transcripts of this multi-copy gene. The hybridization-based approach used in this study allows only for the expression analysis of all *FRG1*- and *FRG2*-like sequences in the genome.

Microarrays are, nevertheless, a valuable tool to analyse simultaneously the expression pattern of all genes within a particular region, such as 4q35 in FSHD. Thus, we can test the hypothesis of a regional position effect in FSHD muscle. Strikingly, we found that none of the single-copy genes localizing to the FSHD region are significantly altered in expression, both by Affymetrix and custom cDNA microarray *(Table 23.3).* On the Affymetrix GeneChip, eight FSHD region (4q35) genes are present on both the HuFL and U95A arrays, with an additional seven genes present only on the U95A GeneChip. Of the single-copy genes in the 4q35 FSHD region (*FACL2, ANT1, IRF2, KLKB1, FAT, CASP3, F11, TLR3, ALP, ING1L, ARGBP2, DCTD* and *DKF2P564J102*), none exhibited a significantly altered pattern of expression in FSHD. The 4q35 gene *ANT1* (SLC25A4) was found to be slightly elevated in FSHD (1.32/1.2-fold elevation on the HuFL and U95A arrays, respectively). However, this increase was not statistically significant at $P<0.05$.

The custom FSHD cDNA array contains many of the single-copy, characterized genes localizing to 4q35 that were analysed by the GeneChip oligonucleotide expression array. Significantly, none of the FSHD region genes analysed using this cDNA array platform were dysregulated in FSHD muscle, supporting the oligonucleotide array data. The cDNA array contained several non-4q35 genes shown to be

Table 23.3 Expression of single-copy 4q35 genes in FSHD muscle

Probe set		Gene description	Symbol	HuFL	U95A	cDNA
HuFL	U95A			FC	FC	FC
D10040	40082	fatty-acid-coenzyme A ligase	*FACL2*	1.9	1.1	1.6
J04982	32822	adenine nucleotide translocator 1	*ANT1*	1.3	1.3	0.9
X15949	1220	interferon regulatory factor 2	*IRF2*	1.2	1.1	1.3
L76159	38923	FSHD region gene 1	*FRG1*	1.1	−1.2	0.7
M13143	32353	kallikrein B1 (Fletcher factor)	*KLKB1*	1.3	1.0	0.8
X87241	40454	FAT tumour suppressor	*FAT*	1.3	1.4	1.4
U13737	36143	caspase 3	*CPP32*	1.6	1.3	0.7
M20218	35591	coagulation factor XI	*F11*	−1.0	−21.3*	0.9
na	31686	tubulin, beta polypeptide 4Q	*TUBB4Q*	na	1.0	na
na	33488	toll-like receptor 3	*TLR3*	na	−1.3	1
na	39690	α-actinin-2-associated LIM protein	*ALP*	na	1.2	1.5
na	39554	inhibitor of growth family, 1-like	*ING1L*	na	1.0	1
na	39295	Arg/Abl-interacting protein	*ARGBP2*	na	−1.9	1.2
na	630	dCMP deaminase	*DCTD*	na	–1.2	na
na	34423	DKF2P564J102 protein	*DKF2P564J102*	na	3.2	na
na	na	FSHD region gene-2	*FRG2*	na	na	1.2
na	na	melatonin receptor Type 1A	*MTNR1A*	na	na	NE
na	na	double homeodomain, D4Z4	*DUX4*	na	na	NE
na	na	hydroxyprostaglandin dehydrogenase 15-(NAD)	*HPGD*	na	na	1

FC=Fold change in expression level (FSHD/Normal); na=not applicable, sequence not present on microarray; NE=not expressed.
Expression values >0 were present in fewer than three samples for each experimental group. Fold change values for these transcripts are not accurate.

dysregulated on the GeneChip, such as MLP, limatin and lumican, which were used as internal controls. All these genes displayed similar changes on the cDNA array, confirming the ability of this array to identify differential gene expression (Winokur *et al.*, 2003b).

Thus, microarray analysis does not support a region-wide position effect hypothesis in FSHD. While *FRG1* and *FRG2* may be dysregulated in FSHD, such a position effect does not appear to extend throughout the FSHD gene region. This conclusion is supported by at least one other study examining both RNA and protein expression of the single copy 4q35 gene *ALP* (Bouju *et al.*, 1999). Recent data utilizing the high-density U133 GeneChip also do not provide evidence for altered expression of single-copy genes in the FSHD region (Yi-Wen Chen, unpublished data). The global disruption of genes involved in myogenesis may therefore occur downstream of *FRG1* and *FRG2*. An alternative model for FSHD pathogenesis may involve mislocalization of the D4Z4/4qtel region within the nucleus. Proper nuclear positioning, as discussed below, is essential for normal gene regulation.

23.4 Myoblasts as models for differentiation

23.4.1 Myogenesis

Global gene expression profiling in FSHD muscle has indicated a defect in myogenic differentiation. Many genes involved in myogenesis, differentiation and cell cycle control as well as several MyoD target genes were dysregulated in an FSHD-specific manner (Bergstrom *et al.*, 2002; Winokur *et al.*, 2003b). Skeletal muscle cell differentiation is a highly ordered sequential process involving the expression of myogenic transcription factors followed by up-regulation of the cyclin-dependent protein kinase inhibitor p21, cell cycle arrest, muscle-specific protein expression and cell fusion to form multinucleated myotubes (Walsh and Perlman, 1997; Seale *et al.*, 2001). Muscle differentiation is orchestrated by two families of myogenic transcription factors, MEF1 and MEF2 (Yoshida *et al.*, 1998; Naya and Olsen, 1999; Perry and Rudnicki, 2000). The MEF1 family, or myogenic bHLHs, consists of MyoD, Myf-5, myogenin and MRF4. MyoD and Myf-5 play redundant roles in specifying muscle lineage (formation of myoblasts), whilst myogenin is required for terminal differentiation and MRF-4 for muscle fibre maturation. MEF2 cooperates with the myogenic bHLHs in activating muscle transcription and differentiation.

Methods for the propagation and differentiation of muscle satellite cells as primary myoblast cultures have allowed investigation of this programme of muscle development (Rando and Blau, 1994; Seale and Rudnick, 2000). Myoblasts in culture mimic many features of myogenesis. When grown in complete media with growth factors and fetal bovine serum, myoblasts proliferate without undergoing differentiation. When 60–80% confluent cells are subjected to serum starvation, they fuse into multinucleated myotubes over a period of several days. These myotubes express muscle-specific transcripts characteristic of mature muscle *in vivo*, such as myogenin and embryonic myosin heavy chain.

23.4.2 Gene expression in FSHD myoblasts

Since experimental evidence generated by microarray analysis indicates a defect in the differentiation process in FSHD, we have carried out expression studies in FSHD and

control myoblasts. As detailed above, myoblasts in culture can mimic the process of myogenic differentiation and thus great insight can be gained through the study of these cells from disease and normal tissue. The investigation of FSHD myogenesis has involved both miroarray analysis of myoblasts, and protein expression studies over a time course of differentiation from myoblasts into multinucleated myotubes.

Initial gene expression studies were performed on FSHD, normal and disease control myoblasts prior to differentiation into myotubes (Winokur *et al.,* 2003a). Undifferentiated myoblasts from FSHD patients exhibit a phenotype that is morphologically distinct from normal myoblasts. The FSHD myoblasts exhibit a 'Vacuolar/necrotic' phenotype, with swelling of the nucleus and the cytoplasm, and vacuolation (Figlewicz *et al.,* 2002; Winokur *et al.,* 2003a; Chapter 16). The necrotic/vacuolar phenotype of FSHD myoblasts indicates that aberrant gene expression may occur early on in FSHD skeletal muscle development. Indeed, gene expression profiling experiments on FSHD myoblasts revealed dysregulation of genes involved in two categories: extracellular matrix formation and oxidative stress (Figlewicz *et al.,* 2002; Winokur *et al.,* 2003a; Chapter 16).

A large number of proteins involved in the formation of the ECM were down-regulated at the RNA level in FSHD myoblasts. Of particular note were the extra-cellular small proteoglycans decorin, lumican and aggrecan, as well as collagen type III, α1. This consistent down-regulation in FSHD myoblasts is seen relative to both normal and disease controls. Elastin *(ELN)* gene transcription is also distinctly different in FSHD muscular dystrophy, as it was down-regulated in every FSHD myoblast culture, and may be related to the morphological changes in these cells. In addition, transcription of the tissue metalloproteinase *TIMP3* gene, was consistently decreased in FSHD. A similar decrease in *TIMP3* was not observed when disease controls were compared to normal myoblasts. This is of particular interest because matrix metalloproteinases have recently been found to play a role in the invasion of T lymphocytes in the inflammatory myopathies (Kieseier *et al.,* 2001). Since FSHD can have a distinct inflammatory component (Arahata *et al.,* 1995), the involvement of TIMP3 should be further investigated in this disease.

Many genes involved in the buffering of cells to the effects of free radical production and oxidative stress are also dysregulated in FSHD myoblasts (Winokur *et al.,* 2003a). These include glutathione S-transferase theta 2, glutathione reductase and heat shock protein 70. Acute exposure to the redox cycling compound paraquat, which induces free radical production, revealed that FSHD myoblasts are in fact more susceptible to oxidative stress than either normal or disease control cells (Figlewicz *et al.,* 2002; Winokur *et al.,* 2003a; Chapter 16). Myoblasts were exposed to varying concentrations of paraquat over 18–24 hours to evaluate their resistance to oxidative stress. Little change in morphology was noted after 24 hours for normal and disease controls, even at the highest concentrations of paraquat. Most cells retained normal morphology, although a small percentage of cells exhibited a vacuolar/necrotic phenotype similar to that seen in FSHD cells cultured in growth media with no oxidative stressor. By contrast, FSHD myoblasts exhibited a pronounced necrotic phenotype at even the lowest concentration of paraquat. FSHD muscle may thus be especially vulnerable to oxidative stress, as this is the only muscular dystrophy so far to demonstrate enhanced susceptibility to oxidative stress in undifferentiated myoblasts.

23.4.3 Protein expression in FSHD myoblasts

FSHD myoblasts in culture are seen to fuse into myotubes at a faster rate than controls, suggesting that the muscle differentiation programme has been turned on at an earlier, perhaps premature timepoint (Figlewicz *et al.*, 2002; Winokur *et al.*, 2003a; Chapter 16). This premature fusion of FSHD myoblasts is not due to replicative senescence of these cells in diseased muscle, as quantitation of telomeric/centromeric repeat ratios do not differ between FSHD and normal myoblasts (Figlewicz *et al.*, 2002; Winokur *et al.*, 2003a; Chapter 16). Interestingly, alteration in D4Z4 copy number has been shown to directly affect myogenic differentiation in C2C12 myoblasts (Yip and Picketts, 2003). Deformed myotube morphology and a reduced myotube fusion index result from increasing numbers of D4Z4 repeats. While disease severity is inversely correlated with the number of D4Z4 repeats in FSHD, the demonstration that D4Z4 can affect myogenesis in *trans* provides further support for a disruption of this process in FSHD.

As described earlier, gene expression profiling experiments indicate that FSHD muscle experiences deficiencies in myogenesis. FSHD may result from the inappropriate triggering of myogenic gene transcription prior to the normal onset of differentiation. Alternatively, since FSHD is an adult-onset muscular dystrophy, altered myogenesis in FSHD may result from inefficient termination of the myogenic programme once differentiation has occurred. The continued expression of genes involved in differentiation could interfere with proper function of mature muscle. Our recent data following muscle-specific protein expression throughout a time-course of myoblast differentiation suggest that the former hypothesis is likely to be correct.

Immunohistochemical analysis of several proteins involved in myogenesis, i.e. myogenin, myosin heavy chain (MHC) and the proliferation marker Ki-67 (Schmidt *et al.*, 2002), was performed in both FSHD and control myoblast cultures. Proliferating cultures were grown in SkGM media (BioWhittaker), 20% fetal bovine serum at 37°C, 5% CO_2. Cells were passed prior to reaching 60–80% confluency, so that fusion into multinucleated myotubes (differentiation) was not induced. FSHD and control myoblasts were then plated at equal densities onto laminin-coated slides. Once culture dishes were 90% confluent, differentiation was induced by switching to DMEM with 2% horse serum (0 hours). The percentage of cells expressing myogenin and Ki-67 at 0, 24 and 72 hours post-differentiation was counted. The fusion index was also determined by calculating the number of nuclei found within MHC- positive myotubes relative to the total number of nuclei at 72 hours.

FSHD and normal myoblasts initially exhibit similar proliferative rates as measured by immunofluorecence of Ki-67 on Day 0. However, upon serum starvation (differentiation media switch), rates of proliferation decrease dramatically in FSHD myoblasts relative to normal myoblasts at 24 hours. Concomitantly, myogenin expression in the FSHD myoblasts is elevated at 24 hours relative to controls. At 72 hours, following the onset of differentiation, the myotube fusion index was greater in FSHD myotubes (47%) than in normal myotubes (32%). Taken together, these data lend support to the hypothesis that FSHD myoblasts undergo an inappropriate response to differentiation signals.

23.4.4 Nuclear localization studies

Although a gradient of altered expression throughout 4q35 has been proposed (Gabellini *et al.*, 2002), expression profiling of FSHD muscle clearly does not support the region-wide position effect model for this disease. Examination of single-copy genes throughout the 3–4 Mb region proximal to D4Z4 does not reveal an aberrant pattern of gene expression. Utilizing both the oligonucleotide and cDNA microarrays, the only single-copy gene in the region found to be slightly up-regulated (although not to a statistically significant degree) in FSHD was *ANT1* by an average of 1.3-fold. None of the other single copy genes *(Table 23.3)* were found to be altered by RNA expression analysis. Thus, while *FRG1* and *FRG2* may be up-regulated in FSHD (Gabellini *et al.*, 2002), none of the single-copy genes in the FSHD region are altered, contrary to the long-held belief that FSHD may result from altered expression of multiple genes throughout the FSHD gene region via a position effect.

Perhaps the FSHD-associated changes in subtelomeric chromatin structure affect the global expression of genes involved in myogenic differentiation rather than regional disruption of 4q35 genes. This may arise through altered nuclear localization of the FSHD region, in turn disrupting proper regulation of genes elsewhere in the genome. Appropriate nuclear localization is essential for normal gene expression (Cremer and Cremer, 2001). Telomeric regions are known to localize to discrete nuclear domains and to regulate the expression of genes within this domain (Francastel *et al.*, 2000; Baur *et al.*, 2001). Proper nuclear localization is also essential for normal DNA replication and cell division (Stein *et al.*, 1996; Leitch, 2000). This may explain the disruption of multiple genes involved in the cell cycle and differentiation seen upon expression profiling of FSHD muscle.

Since the 4q telomere localizes to the nuclear periphery (our unpublished data), the association of D4Z4 with the nuclear envelope is an area of active research. Disruption of the nuclear envelope in other forms of muscular dystrophy is well established (Burke and Stewart, 2002). Emery-Dreifuss muscular dystrophy (EDMD) is caused by mutations in the genes for either emerin or lamin A, both essential for the proper formation of the nuclear envelope. The clinical phenotype is believed to result either from nuclear fragility or alterations in global gene regulation mediated by disorganization of heterochromatin and transcription factor binding (Maraldi *et al.*, 2002). In support of the latter hypothesis, other diseases exhibiting a broad array of symptoms are also due to mutations in lamin A. These include familial partial lipodystrophy, Hutchinson-Gilford progeria, Charcot-Marie-Tooth disease and mandibuloacral dysplasia (Cao and Hegele, 2003; Ostlund and Worman, 2003). Interestingly, we have recently shown that D4Z4 associates with the nuclear matrix (our unpublished data). Such an association strengthens the argument that the D4Z4 repeat has a role in the structure and organization of chromatin and perhaps, as a result, the regulation of gene expression. Since lamin A is a component of both the nuclear envelope and the nuclear matrix (Hutchinson, 2002), this effect may be mediated by the interaction of D4Z4 repeats with lamin A. FSHD may result from a disruption of this interaction, perhaps in itself mediated by altered methylation or histone modification of the deleted D4Z4 repeat tract.

23.5 Summary

Although the precise mechanism of gene dysregulation in FSHD remains unknown, the disease is not quite the 'black box' that it was just a few years ago. Great strides have been made in understanding the pathogenesis of FSHD, from specific alterations in chromatin structure, to their consequent misregulation of gene expression both at 4q35 and elsewhere in the genome, to the cellular pathways affected in myoblasts and muscle tissue.

Many questions have yet to be answered in order to fully understand the basis of FSHD and before appropriate therapies can be designed. Firstly, are the 4q35 genes *FRG1* and *FRG2* primarily responsible for the phenotype? If so, what are the precise functions of these genes? Animal models may help to address this issue. Secondly, are genes elsewhere in the genome directly affected by deletions of D4Z4 at 4qter, perhaps by altered nuclear localization? If so, what are these genes, and what is the mechanism behind their misregulation? Perhaps lessons can be learned from other diseases such as EDMD. Thirdly, can we design therapies for cellular pathways that appear to be affected in FSHD, such as the increased susceptibility to oxidative stress, and the propensity for FSHD muscle cells to respond inappropriately to differentiation signals? Careful investigation of these possibilities is sure to reap benefits for those affected by this fascinating and unusual disorder.

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24.
Therapeutic trials and medical management in FSHD

Rabi Tawil and Robert C.Griggs

FSHD Facioscapulohumeral Muscular Dystrophy: Clinical Medicine and Molecular Cell Biology, edited by Meena Upadhyaya and David N.Cooper.

24.1 Introduction

The explosion of molecular genetic information has provided tangible new targets for therapeutic interventions in a number of muscular dystrophies. Yet, despite this wealth of genetic discoveries, no new effective therapeutic interventions exist for the treatment of muscular dystrophies. Moreover, the enticing promise, yet to be fulfilled, of gene therapy has led to the relative neglect of research into potential pharmacologic interventions.

In striving to find effective treatments, FSHD offers several advantages, as well as one major disadvantage, over some of the other dystrophies. The one disadvantage is that, despite advances in our understanding of the molecular defect in FSHD, the gene or genes that mediate the effects of the deletion remain unknown. Consequently, without knowledge of the pathophysiology of FSHD, it is difficult to devise rational, targeted treatment approaches.

On the other hand, FSHD has several advantages over other muscular dystrophies when considering the conduct of therapeutic trials:

1. FSHD is a relatively common dystrophy with clearly defined clinical and genetic diagnostic criteria making recruitment of patients straightforward.
2. FSHD has few clinically important extramuscular manifestations that would preclude the use of certain pharmaceutical agents.
3. The biggest advantage, however, is the fact that, other than Duchenne, FSHD is the only dystrophy whose natural history has been prospectively studied and in which sizable randomized controlled trials have been performed (Personius *et al.*, 1994; Tawil *et al.*, 1994; The FSH-DY Group, 1997).

Direct and surrogate measures of disease activity have been tested and crucial information about disease progression has been learned to allow accurate power

calculations. The information obtained from these studies is crucial for the effective design and implementation of future therapeutic trials in FSHD. We have out- come measures for therapeutic trials and we know the numbers of patients needed for a clinical trial of any prespecified duration.

24.2 Therapeutic trials in FSHD

24.2.1 Corticosteroids

Mononuclear inflammatory infiltrates are seen in as many as 40% of muscle biopsy samples of patients with FSHD (Padberg, 1982). At times this inflammation is intense enough to suggest an inflammatory myopathy (Bates *et al.*, 1973). Case reports of FSHD patients treated with corticosteroids showed either improvement or no effect (Munsat *et al.*, 1972; Bates *et al.*, 1973; Wulff *et al.*, 1982). Some the patients treated with prednisone initially improved in strength (Munsat *et al.*, 1972), though this improvement quickly waned and progression resumed (Munsat and Bradley, 1977). As a consequence of these early reports, it became relatively common practice to give FSHD patients with inflammatory biopsies a trial of corticosteroids. Such a practice was bolstered by the clear demonstration of the therapeutic benefits of prednisone in boys with Duchenne muscular dystrophy (Mendell *et al.*, 1989; Griggs *et al.*, 1991).

The effects of prednisone on FSHD were subsequently studied in a prospective, 3 month, open-label trial (Tawil *et al.*, 1997). Prednisone (1.5 mg/kg/day) was administered for 12 weeks to eight patients with FSHD. There were no significant changes in measures of muscle strength or muscle mass (Tawil *et al.*, 1997). Given the limited power of this study, no conclusions regarding the effects of prednisone in slowing or arresting disease progression could be reached. However, the authors concluded that no further trials with prednisone could be justified given the risk versus limited benefit of lifelong use of corticosteroids (Tawil *et al.*, 1997).

24.2.2 Albuterol

Compounds that demonstrate skeletal muscle anabolic properties are logical putative therapeutic agents for any muscle-wasting disease. One such class of compounds is the beta-adrenergic agents. β_2 agonists exert a number of effects on muscle metabolism and function including proliferation of satellite cells, increased muscle protein synthesis and inhibition of muscle proteolysis (Benson *et al.*, 1991; Matlin and Delday, 1992; Matlin *et al.*, 1992). Protective effects of these compounds have been demonstrated in animal models of denervation, sepsis, and corticosteroid atrophy as well as animal models of muscular dystrophy (Zeman *et al.*, 1987; Choo *et al.*, 1989; Benson *et al.*, 1991; Agbenyega and Wareham, 1992; Dupont-Versteegden, 1996; Hayes and Williams, 1998). Human studies, in normal volunteers, have also demonstrated the anabolic effects of the β_2 agonists (Greenhaff, 1997; Kissel *et al.*, 1998, 2001). Clenbuterol, a potent β_2 agonist, is considered a performance-enhancing drug and is banned by the Olympics authorities (Winter and Spann, 1995).

A positive, 3-month, open-label trial of a sustained-release albuterol in FSHD led to the conduct of a 1-year, randomized, placebo-controlled trial (Kissel *et al.*, 1998, 2001). In this study, 90 FSHD patients were enrolled and randomized to receive two different doses of sustained-release albuterol (8 mg b.i.d. and 16 mg b.i.d.). Although a trend towards improved muscle strength was seen at 3 months in those groups receiving active drug, no difference between the treatment groups was noted at 1 year. Interestingly, muscle mass, as estimated by measurement of dual energy X-ray absorptiometry, was significantly increased at 1 year in the active drug groups *(Figure 24.1)* (Kissel *et al.*, 2001). The authors speculated that the increase in muscle mass (mean: 1.7–1.9 kg), was insufficient to translate into measurable improvement in overall muscle strength. The peak of the improvement in muscle mass was noted at the 3-month follow-up period. A similar study of albuterol, combined with an exercise regimen was completed in the Netherlands but the results have not yet been published.

24.2.3 Creatine monohydrate

Creatine supplementation has recently received attention in the treatment of muscle-wasting diseases. How creatine-P, which is the immediate source of energy during vigorous muscle contraction, can improve strength in longstanding dystrophic conditions remains unclear. Creatine supplementation in normal athletes at best, slightly enhances performance during high-intensity, short-term exercise (Greenhaff, 1997; Williams and Branch, 1998). There is evidence that phosphocreatine stores are depleted in some dystrophic muscle and that creatine may have cellular protective characteristics (Kemp *et al.*, 1993; Pulido *et al.*, 1998). One randomized double-blind, cross-over trial, in a mixed population of dystrophies, including 12 patients with FSHD, demonstrated slight improvement in overall strength following short-term (8-week) supplementation with creatine monohydrate (Walter *et al.*, 2000). This improvement was reflected both in the neuromuscular symptom score as well as in changes in composite MRC scores. Given the size of the trial and the mixed subject population, little can be concluded as to the utility of creatine supplementation in FSHD.

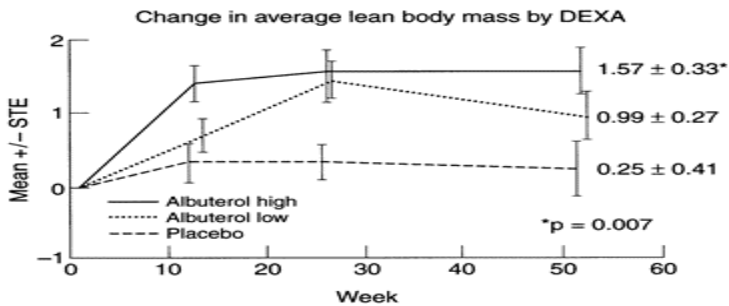

Figure 24.1 Change in lean body mass at 52 weeks as determined by dual energy X-ray absorptiometry (DEXA) scanning. The high-dose albuterol

group (uppermost line) showed a significant increase in lean mass compared to placebo (bottom line) (P=0.007). The low-dose group (middle line) also gained lean mass, but it was not significant compared to placebo (P=0.08).

24.3 Medical management

In the absence of effective therapeutic intervention in FSHD, medical management is aimed at maintaining the individual's functional capabilities and at providing symptomatic treatment of associated complaints.

24.3.1 Assistive devices

In general, because FSHD is slowly progressive, affected individuals develop effective adaptive strategies to compensate for their disabilities, often without necessitating assistive devices. Ankle-foot orthoses are useful for foot drop, particularly early in the course of the disease. If significant quadriceps weakness sets in, however, fixed ankle-foot orthosis can hinder ambulation by preventing hyperextension and locking of the knee. In such instances the use of floor reaction ankle-foot orthoses (FRAFO) is advisable. The FRAFO, with its anterior tibial lock provides extension force to the knee upon floor contact, preventing buckling of the knee.

24.3.2 Surgical interventions

Surgical scapular fixation, in a select group of patients, can significantly enhance arm mobility (Bunch and Siegel, 1993; Copeland *et al.,* 1999). Several factors have to be considered before recommending surgery. One is whether fixation will actually result in greater arm movement in a given patient. This is easily tested at the bedside by assessing the degree of functional gain when the scapulae are manually fixed by the examiner. The second factor is the rate of progression in the individual patient. If the disease is rapidly progressive, the benefit of surgery may be short-lived. Shoulder range of motion becomes more restricted with scapular fixation especially when done bilaterally. Patients should be aware of this limitation before considering bilateral scapular fixation. Tendon transfer procedure for foot drop is another potentially useful intervention for patients with FSHD. As with scapular fixation, it should be reserved for patients who demonstrate slow progression with sparing of the calf muscles.

24.3.3 Role of exercise

The utility of prescribing an exercise regimen to patients with any form of muscular dystrophy remains an unresolved and controversial issue. The major source of concern is

the potential deleterious effects of exercise in patients with dystrophies, such as Duchenne, where structural compromise of the muscle membrane renders muscle fibres more susceptible to contraction injury. No such abnormality has been demonstrated or suspected in FSHD. Moreover, small, short-term studies of exercise in FSHD demonstrate benefit (Vignos and Watkins, 1966; McCartney *et al.*, 1988; Milner-Brown and Miller, 1988). It is our practice to encourage patients with FSHD to remain physically active. The intensity of allowable exercise is tailored to the individual patient's overall strength with the general goal of emphasizing aerobic conditioning and toning over muscle-building exercises.

24.3.4 Pain

Pain is not typically thought of as a major manifestation of FSHD. However, a recent paper highlighted the presence of significant pain in a number of patients (Bushby *et al.*, 1998). Moreover, in a recent survey done by the AFM (Association Francaise Contre les Myopathies), a substantial percentage of patients with FSHD complained of pain (unpublished). Much of the pain is musculoskeletal in origin and is due to changes in posture and laxity of joints resulting in low back, neck and shoulder pain. Such pain can be managed with established protocols for the treat ment of chronic musculoskeletal symptoms.

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Appendix I
The FSH Society

Daniel Perez

The FSH Society: a partnership of patients, families, clinicians and scientists

The Facioscapulohumeral Society (FSH Society) is a privately funded not-for-profit organization. From its inception, the FSH Society has been patient-driven. The idea of creating a mechanism to provide FSHD-affected persons and their families with FSHD-related information and to promote FSHD-specific scientific and clinical research was conceived in 1988 by two men affected by the disease, Stephen J.Jacobsen, Ph.D., and the author, Daniel Paul Perez. Dr. Jacobsen was already in the process of establishing a national FSHD cell bank, and contacts with persons involved in his study led to the development of a grass-roots organization dedicated to FSHD issues. A Board of Directors was created and donations were solicited. By late summer of 1993, thanks to generous donations of time and money from FSHD-affected persons and their families and friends, the FSH Society was a fully functioning organization.

In accordance with its primary purpose of serving the FSHD community, both in the United States and abroad, the FSH Society, through outreach at home and international networking, has brought together more than 3000 FSHD-affected families committed to working cooperatively. From the moment of their introduction into the FSH Society, these families, and, in many instances, their friends, are bonded with their fellow members both by their common knowledge of what it is to live with FSHD and by the ardent desire they all feel to be part of a concerted effort to discover how to treat the disease and, ultimately, to cure it.

People who have FSHD must cope with continuing, unrelenting, unpredictable and never-ending losses. The most unlucky, those who are affected from birth, are deprived of virtually all the ordinary joys and pleasures of childhood and adolescence. But no matter at which stage of life the disease makes itself known, there is never, after that, any reprieve from continuing loss of physical ability, or ever for a moment relief from the physical and emotional pain that FSHD brings in its train. Every morning, FSHD sufferers wake up to face the reality that neither a cause for their disease nor any treatment for it has yet been found.

Insidiously and systematically, FSHD denies a person the full range of choices in life. FSHD affects the way you walk, the way you dress, the way you work, the way you

wash, the way you sleep, the way you relate, the way you parent, the way you love, the way and where you live, and the way people perceive and treat you. You cannot smile, hold a baby in your arms, close your eyes to sleep, run, walk on the beach, or climb stairs. Each new day brings renewed awareness of the things you may not be able to do the next day. This is what life is for tens of thousands of people affected by FSHD worldwide.

Through the FSH Society, FSHD patients have found ways to be useful to medical and clinical researchers working on their disease. The FSH Society acts as a clearinghouse for information on the FSHD disorder and on potential drugs and devices designed to alleviate its effects. It fosters communication among FSHD patients, their families and caregivers, charitable organizations, government agencies, industry, scientific researchers, and academic institutions. It solicits grants and contributions from members of the FSH Society, and from foundations, the pharmaceutical industry, and others to support scientific research and development. It makes grants and awards to qualified research applicants. In less than 5 years, the FSH Society has raised more than $1.1 million for research and has invested it in two dozen innovative research programs internationally (see below). One of the FSH Society's key assets, its Scientific Advisory Board, is composed of international experts whose awareness of current FSHD research ensures both that new research is not duplicative but complementary and that it will fill gaps in existing knowledge. The FSH Society's work in education, advocacy, and training has led to increased funding in the United States and abroad. It was a key participant in drafting the Muscular Dystrophy Community Assistance Research and Education Act of 2001 (MD CARE Act), which in the United States mandates research and investigation into all forms of muscular dystrophy.

Ten years ago, when Dr. Jacobsen and I set out to seek solutions to the tangled puzzle of FSHD, we knew that we had embarked on a difficult and risky journey. But we quickly learned that a striking aspect of the FSHD patient population is its high levels of intelligence and professional achievement, and that these make it possible for the patient and medical communities to engage in an ongoing, challenging, and productive dialogue. The FSH Society-sponsored patient, researcher, and combined patient/researcher network conferences are the centrepiece of this dialogue. The FSH Society is proud that many of the contributors to this book are FSH Society Fellows and members of its Scientific Advisory Board.

The FSH Society and its members salute all of the researchers whose papers are presented here. Your expertise, dedication, and provocative thinking have resulted in exciting, promising progress towards the goal of fully understanding the FSH disease and its causes. You have brought us hope. We all take great satisfaction in knowing that we have been of help to many of you, and, both as a Society and as individuals, we look forward to continuing to act as catalysts working side-by-side with you. We are your friends, your neighbours, your colleagues. We hope that you will continue to walk/roll with us.

Daniel Paul Perez
President and CEO
FSH Society, Inc.

Patients, professionals, and other parties interested in FSHD can assist our efforts by joining us. Please contact us at FSH Society, Inc., 3 Westwood Road, Lexington, MA 02420, USA. Phone (+001781) 860-0501, fax (+001781) 860-0599, e-mail: daniel.perez@fshsociety.org. Internet: http://www.fshsociety.org/.

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Project Title:	“Generation of transgenic mouse models for FSHD."
Researcher:	Sara T.Winokur, Ph.D.
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Project Title:	“Analysis of chromatin structure and skeletal muscle-specific gene.”
Researcher:	Denise Figlewicz, Ph.D.
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Project Title:	“Expression of genes proximal to the D4Z4 deletions: a quantitative study in FSHD patients and controls.”
Researcher:	David J.Picketts, Ph.D.
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Project Title:	“Utilizing an epigenetic approach to identify the FSHD gene.”
Researcher:	Davide Gabellini, Ph.D.
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Project Title:	“Identification and characterization of a protein interacting with the DNA repetitive element causally related to facioscapulohumeral muscular dystrophy.”
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Project Title:	"Characterization of a protein expressed from a 3.3 kb element not linked to FSHD" & "Small laboratory equipment for research on FSHD."
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Project Title:	"Characterization of differentially expressed genes in facioscapulohumeral muscular dystrophy affected muscles."
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Project Title:	"*Fugu rubripes* as a model organism for FSHD gene identification."
Researcher:	Marcy Speer, Ph.D.
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Project Title:	"Genetic linkage studies in non-chromosome 4 FSHD."
Researcher:	Robert Bloch, Ph.D.
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Project Title:	"Sarcolemmal organization in FSHD and the MYD mouse" & "To investigate the 'proteome' in FSHD and to compare it to the 'proteome' in control muscles and in other common myopathies and muscular dystrophies using two-dimensional gel electrophoresis"
Researcher:	Kevin Flanigan, M.D.
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Project Title:	"QMA software/system and professional physical therapy resources to help with studies to answer definitively whether anticipation in disease severity and onset, gender effects, or parent-of origin effects exist in FSHD."
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Researcher:	York Marahrens, Ph.D./Nieves Embade, Ph.D.
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Project Title:	"Tethering adenine (Dam) methylase to the 3.3-kb FSHD repeats to identify distant genes that physically come in contact with the repeats"
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Appendix II
The Muscular Dystrophy Campaign: pioneering research, providing care

It is estimated that muscular dystrophy and related conditions affect around 30000 people in the UK with a further 120 000 people impacted—carriers of the genes, carers, family members, etc. There are over 60 different types of these muscle wasting and weakening conditions which fall under the umbrella of the Muscular Dystrophy Campaign and the charity is the only one in the UK covering the wide range of disorders.

The Muscular Dystrophy Campaign was formed over 40 years ago and exists to improve quality of life for people with neuromuscular disorders and to provide hope for the future.

Pioneering research

Throughout its history the Muscular Dystrophy Campaign has been a key motivator in pioneering the search for a cure and continues to play a major role in helping the worldwide scientific effort. The charity manages a portfolio of grants aimed at gaining a better understanding of the conditions with the ultimate aim of identifying treatments and cures. Around £1.5 million is spent every year with 30 projects at some of the UK's leading universities and hospitals.

The first gene implicated in muscular dystrophy, the dystrophin gene, was identified in 1986 and linked to Duchenne and Becker muscular dystrophy. To date the vast majority (around 90%) of genes involved in these conditions have been identified. Once the disease process is understood research moves towards exploring therapeutic options. This stage has been reached for several of the muscular dystrophies and involves strategies such as pharmacological agents, gene therapy and cell therapy. For some conditions it has not been possible to link a known mutation with a gene(s) and more effort has been required to gain an understanding of how the mutation causes the disorder. In other cases the condition is not caused by a genetic mutation and some have been linked to a faulty immune system.

The Muscular Dystrophy Campaign also funds Muscle Centres, centres of excellence providing both a clinical service to individuals and a platform for research. Research departments affiliated with Centres have access to genetic data and samples taken from

those affected, which allows research results to be quickly translated into services such as diagnostic tests.

There is still some way to go, but improvements in diagnosis and the introduction of genetic counselling have been made possible by the charity's commitment to research. Now the charity is looking at a range of potential treatments, including gene therapy, to help people suffering from the condition in the not too distant future.

Providing care

The charity also helps people come to terms with the impact of muscular dystrophy on a day-to-day level. Through a network of Family Care Officers (FCO) the Muscular Dystrophy Campaign provides unparalleled support services across the UK for those who suffer from MD and for their families. These hospital-based FCOs work to give people maximum independence and quality of life. As experts in the disorder they can offer practical advice and information and emotional support to those newly diagnosed. With their help, living with the severe forms of MD can be made more bearable.

The Muscular Dystrophy Campaign also gives adults and children mobility and independence by funding essential equipment such as wheelchairs and computers through the Joseph Patrick Memorial Trust. The charity's national Information Service publishes factsheets on all the major conditions, lifestyle and medical issues and is able to offer advice by telephone, letter or e-mail. In addition, a national occupational therapy advisor provides information on housing adaptations and equipment not only to families but also to health professionals.

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Appendix III
The Association Française contre les Myopathies

The Association Française contre les Myopathies (French Muscular Dystrophy Association) has the goal of supporting and encouraging basic and clinical research on neuromuscular diseases. Created in 1958 by a group of patients and their families, recognized as a public utility in 1976, the AFM (French Muscular Dystrophy Association) has a single objective: to defeat all those neuromuscular diseases that are devastating muscle-wasting diseases. The AFM has set itself two missions: to cure all neuromuscular diseases and to reduce the disabilities they cause.

Since many neuromuscular diseases have a genetic origin, the AFM is involved in the development of scientific tools for the study of genetic and rare diseases in general, and it provides scientists with new means and knowledge to accelerate our understanding of genetic diseases and to open up treatment avenues based on a better understanding of the causative genes (source AFM website).

Within the framework of advancing knowledge about FSHD, the association a year ago established a 'Club FSHD', that is comprised of a number of international research teams working in this field. The aim of the group is to develop a common project in which everyone is involved, dependent on their different areas of expertise. An additional group, Group of Research and Action on FSHD (GRAF) is being established to include those clinicians who are concerned with the clinical features of the FSHD phenotype and FSHD patients.

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