

**Analysis of the translocation events
between the repeat arrays
from chromosomes 4q35 and 10q26 in the
South African facioscapulohumeral muscular
dystrophy (FSHD) population**

BY

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**Analise van translokasies
tussen die herhalingsvolgordes
geleë op chromosome 4q35 en 10q26 in die
Suid-Afrikaanse fasioskapulohumerale
spierdistrofie (FSHD) populasie**

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TO MY PARENTS

ABSTRACT

Facioscapulohumeral muscular dystrophy (FSHD) is a progressive autosomal dominant myopathy, preferentially affecting facial, shoulder girdle and upper-arm muscles. It is one of the most common muscular dystrophies with a prevalence of 1 in 20,000.

The rearrangement that results in FSHD entails a deletion of an integral number of 3.3 kb repeat arrays on chromosome 4q35. A candidate gene for this disorder has, however, yet to be identified. Data suggests a position effect variegation model resulting in FSHD.

Southern blot analysis has led to the identification of an additional locus with 95% homology to 4q35, which was subsequently mapped to chromosome 10q26. This region also undergoes a deletion of 3.3 kb repeat arrays in a similar fashion to 4q35. A deletion fragment located on chromosome 10q is, however, non-pathogenic and does not result in FSHD, but rather compromises accurate molecular diagnoses. Previous data suggested a model for subtelomeric plasticity due to the occurrence of DNA translocations between the 4-type and 10-type arrays. The high degree of homology between these fragments may be the predisposing factor that has led to these translocations being observed in 20% of the Dutch population.

The objectives of this study were to optimise the *Bgl* II – *Bln* I dosage test via a non-radioactive protocol and to verify the presence of translocation events between the 4q and 10q arrays in 80 to 100 individuals selected from South African FSHD families.

Results obtained from this study suggest a lower translocation frequency in the South African FSHD population when compared to previously reported frequencies. However, the small sample size implies that inferring statistical significance would be unrealistic. The turnaround time for molecular FSHD diagnosis has been reduced significantly via the standardisation of this non-radioactive detection protocol.

OPSOMMING

Fasioskapulohumerale spierdistrofie (FSHD) is 'n progressiewe, outosomaal, dominante miopatie, wat hoofsaaklik die gesig-, skouergordel- en bo-armspiere aantast. Dit is een van die mees algemene spierdistrofieë met 'n voorkoms van 1 uit 20,000.

Die DNA herrangskikking wat FSHD veroorsaak, behels die deleisie van 'n aantal 3.3 kb herhalende volgordes op chromosoom 4q35. 'n Kandidaatgeen vir hierdie toestand kon nog nie geïdentifiseer word nie. Data dui op 'n varieerbare-posisie-effek-model wat FSHD veroorsaak.

Southern klad analise het 'n addisionele lokus geïdentifiseer op chromosoom 10q26, wat 95% homologie met 4q35 het. Hierdie area ondergaan ook 'n deleisie van 3.3 kb herhalende volgordes op 'n soortgelyke manier as 4q35. Sodanige deleisie op chromosoom 10q is egter nie patogenies nie en veroorsaak nie FSHD nie, maar bemoeilik eerder die akkurate molekulêre diagnose. Vorige data het gedui op 'n model van subtelomeriese plastisiteit omdat DNA translokasies tussen die 4-tipe en 10-tipe volgordes voorgekom het. Die hoë graad van homologie tussen hierdie fragmente kan die bydraende faktor wees wat veroorsaak dat sodanige translokasies in 20% van die Nederlandse bevolking waargeneem is.

Die doelstellings van hierdie studie was om die *Bgl* II – *Bln* I dosistoets deur middel van 'n nie-radioaktiewe protokol te optimiseer en om die teenwoordigheid van translokasiegebeure tussen 4q- en 10q-volgordes in 80 tot 100 individue in Suid-Afrikaanse FSHD-families te bepaal.

Die resultate wat verkry is uit hierdie studie dui op 'n laer translokasie-frekwensie in die Suid-Afrikaanse FSHD-bevolking in vergelyking met vorige gerapporteerde gevalle. Aangesien die steekproef klein was, is beduidende statistiese gevolgtrekkings nie realisties nie. Die omsettyd vir molekulêre FSHD diagnose is aansienlik verkort deur die standaardisering van hierdie nie-radioaktiewe deteksieprotokol.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations are listed in alphabetical order.

A_{260}/A_{280}	ratio of absorbency measured at 260 nm and 280 nm
AP-SA	alkaline phosphatase-labelled streptavidin
β	beta
B_{ave}	average background intensity
<i>Bgl</i> II	restriction endonuclease isolated from an <i>E. coli</i> strain that carries the cloned <i>Bgl</i> II gene from <i>Bacillus globigii</i> , with recognition site 5'-A↓GATCT-3'
<i>Bln</i> I	restriction endonuclease isolated from an <i>E. coli</i> strain that carries the cloned <i>Bln</i> I gene from <i>Brevibacterium linens</i> , with recognition site 5'-C↓CTAGG-3'
boric acid	boracic acid: H_3BO_3
bp	base pair
c or c	cytosine (in DNA sequence)
°C	degrees centigrade
cDNA	complementary DNA
CEN	centromere
chr	chromosome
cm	centimetre: 10^{-2} metre
CPD-Star ^{®1}	disodium 4-chloro-3-(methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclodecan}-4-yl)phenyl phosphate: $C_{18}H_{19}Cl_2O_7PNa_2$
CSPD ^{®2}	disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricycle [3.3.1. ^{13,7}] decan}-4-yl)phenyl phosphate: $C_{18}H_{20}ClO_7PNa_2$
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
ddH ₂ O	double distilled water
dGTP	2'-deoxyguanosine-5'-triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide triphosphate
dsDNA	double stranded DNA
dTTP	2'-deoxythymidine-5'-triphosphate
<i>Eco</i> RI	restriction endonuclease isolated from an <i>E. coli</i> strain that carries the cloned <i>Eco</i> RI gene from <i>Escherichia coli</i> RY 13, with recognition site 5'-G↓AATTC-3'
EDTA	ethylenediamine tetraacetic acid: $C_{10}H_{16}N_2O_8$
ENMC	European Neuromuscular Centre
EtBr	ethidium bromide (2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide): $C_{21}H_{20}BrN_3$
EtOH	ethanol: CH_3CH_2OH
FISH	fluorescence <i>in situ</i> hybridisation
FRG1	FSHD region gene 1
FRG2	FSHD region gene 2
FSHD	facioscapulohumeral muscular dystrophy
G or g	guanine (in DNA sequence)
gDNA	genomic DNA

¹ CPD-Star[®] is a registered trademark of Tropix Inc., Bedford, MA, U.S.A.

² CSPD[®] is a registered trademark of Tropix Inc., Bedford, MA, U.S.A.

GenBank	GenBank ^{® 1} : United States repository of DNA sequence information
HCl	hydrochloric acid
IQs	intelligent quotients
JSG	juvenile shoulder-girdle
kb	kilo (10 ³) base pair
KCl	potassium chloride
KPL	Kirkegaard & Perry Laboratories
<i>Kpn</i> I	restriction endonuclease isolated from an <i>E. coli</i> strain that carries the cloned <i>Kpn</i> I gene from <i>Klebsiella pneumoniae</i> , with recognition site 5'-GGTAC↓C-3'
μ	micro: 10 ⁻⁶
μg	microgram
μl	microlitre
μM	micromolar
m	milli: 10 ⁻³
M	molar: moles per litre
MD	muscular dystrophy
mg	milligram
MgCl ₂	magnesium chloride
ml	millilitres
mm	millimetre
mM	millimolar
mRNA	messenger RNA
MWM	molecular weight marker
n	nano: 10 ⁻⁹
Na-citrate	citric acid trisodium salt: C ₆ H ₅ Na ₃ O ₇
NaCl	sodium chloride
Na ₂ EDTA	disodium EDTA: C ₁₀ H ₁₄ N ₂ Na ₂ O ₈ .2H ₂ O
NaOH	sodium hydroxide
ng	nanogram
NLS	nuclear localisation signal
nm	nanometre: 10 ⁻⁹ meter
orange G	7-hydroxy-8-phenylazo-1,3-naphthalenedisulfonic acid: C ₁₆ H ₁₀ N ₂ O ₇ S ₂ Na ₂
ORF	open reading frame
%	percent
p	pico: 10 ⁻¹²
p13E-11	probe complimentary to 3.3 kb repeat units at locus D4Z4
PCR	polymerase chain reaction
PEV	position effect variegation
PFGE	pulsed field gel electrophoresis
pH	indicates acidity: numerically equal to the negative logarithm of H ⁺ concentration expressed in molarity
PMACH	progressive muscle atrophy of childhood
pmol	pico mole
P/S	ratio of probe to standard
PTS1	peroxisomal target signal 1
q	long arm of a chromosome
qter	telomeric region of the long arm of a chromosome
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute

¹ Genbank[®] is a registered trademark of the National Institutes of Health, Bethesda, MD, U.S.A.

RSA	relative specific activity
RT-PCR	reverse transcriptase PCR
S _{ave}	average signal intensity of fragments
SDS	sodium dodecyl sulphate: C ₁₂ H ₂₅ NaSO ₄
spermidine	N-[3-Aminopropyl]-1,4-butanediamine: C ₇ H ₁₉ N ₃
SSC	saline-sodium-citrate buffer: 0.15 M NaCl, 15mM Na-citrate, pH 7.0
SSCP	single-strand conformation polymorphism
ssDNA	single stranded DNA
SSPE	saline-sodium-phosphate-EDTA buffer: 0.15 M NaCl, 10 mM NaH ₂ PO ₄ , 1 mM EDTA, pH 7.4
T or t	thymine (in DNA sequence)
T _a	annealing temperature
T _m	melting temperature
Taq polymerase	deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7, from <i>Thermus aquaticus</i> BM, recombinant (<i>E. coli</i>)
TBE buffer	Tris borate-EDTA buffer: 89.15mM Tris ^{®1} (pH 8.0), 88.95 mM boric acid, 2.498 mM Na ₂ EDTA
TE	10 mM Tris-HCl (pH 7.5); 1 mM EDTA
TEL	telomere
Tris	Tris [®] : tris(hydroxymethyl)-amino-methane: 2-amino-2-(hydroxymethyl)-1,3-propanediol: C ₄ H ₁₁ NO ₃
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride: C ₄ H ₁₁ NO ₃ ·H ₂ O
TUBB4Q	human beta-tubulin gene
U	units
UV	ultraviolet
V	volt
Xap I (isoschizomer Apo I)	restriction endonuclease isolated from an <i>E. coli</i> strain that carries the cloned Apo I gene from <i>Arthrobacter protophormiae</i> with recognition site 5'-Pu↓AATTPy-3'
YAC	yeast artificial chromosome
Σ	sum of
■ / ●	male/female: tested FSHD normal
▣ / ●	male/female: FSHD equivocal
□ / ○	male/female: never tested for FSHD (phenotypical status unknown)
■ / ●	male/female: tested FSHD positive
▣ / ♂	male/female: deceased
←	proband

¹ Tris[®] is a registered trademark of Rohm & Haas Company, Philadelphia, PA, U.S.A.

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CHAPTER ONE

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is a dominantly inherited disorder with a reported prevalence of 1 in 20,000 within the Dutch population (Padberg, 1982). It displays differential age of onset and a high degree of variability with regards to expression of the clinical phenotype. The myopathy initially manifests via facial and shoulder girdle weakness and atrophy, which progresses to the pelvic girdle and lower extremities. Patients often present with winged scapulae and tend to adopt a characteristic posture with their shoulders thrust forward.

Manifestation of the disorder is primarily within the second or third decades of life with a penetrance of 95% by the age of 20 (Lunt *et al.*, 1989). FSHD results from a DNA deletion at the D4Z4 locus located on chromosome 4q35 (Wijmenga *et al.*, 1992). This telomeric locus is characterised by an array of tandemly repeated sequences, each being 3.3 kb in length. Unaffected individuals harbour 10-100 copies of these repeat arrays, whilst the affected individuals have less than 10.

The DNA rearrangement is investigated via Southern blot analysis and detected utilising probe p13E-11. Unfortunately, p13E-11 cross-hybridises to several other regions in the genome; the most significant of these being to chromosome 10q26. The region displays 95% homology to 4q35. Moreover, 10q26 also displays similar DNA rearrangements to those resulting in the FSHD phenotype. Since the deletions at chromosome 10q26 are non-pathogenic, fragments from this particular locus severely compromise molecular diagnosis of FSHD. Sequence comparison of the two regions revealed unique restriction enzyme sites that could be exploited to allow for differential analyses. The recognition of a *Bln* I site within each of the repeats originating from chromosome 10 (Deidda *et al.*, 1996), complemented by a *Xap* I site in each of the 4-type repeats (Lemmers *et al.*, 2001) thus allowed for complete elucidation of the pathogenic versus non-pathogenic alleles.

The high degree of homology between the 4q35 and 10q26 regions has been proposed to be responsible for the occurrence of translocation events between these repeat arrays (Van Overveld *et al.*, 2000), resulting in 4-type repeats residing on chromosome 10 and

vice versa. Fortunately, Van der Maarel *et al.* (1999) developed a technique, referred to as the *Bgl* II – *Bln* I dosage test that enabled characterisation of these translocation events. The frequency of translocation has been reported to be in the range of 20 to 30% in the various populations investigated to date (Van der Maarel *et al.*, 1999 and Matsumura *et al.*, 2002). Chapter two provides a detailed overview of the FSHD literature.

The South African FSHD population has recently been described on a genetic level (Van der Merwe, 2002). Two deletion fragments of 24 kb and 34 kb have been demonstrated to segregate in the South African FSHD families investigated. Haplotype analyses were also performed to verify the inheritance and regional genetic background of the above deletion fragments. However, the study presented here investigated the occurrence and frequency of translocation events in the South African population. This is the first investigation of its kind in this population. Non-radioactive protocols were adopted in this study to investigate the efficiency of this process in an effort to minimise the turnaround time for FSHD diagnosis.

CHAPTER TWO

AETIOLOGY OF FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

Facioscapulohumeral muscular dystrophy (OMIM 158900) belongs to a family of disorders that primarily affect the muscles of the human body. These disorders have, however, previously been reported to be accompanied by extra-muscular symptoms. The term used to describe this form of muscular dystrophy raised controversy in the past. Several authors referred to it as the “FSHD of Landouzy and Dejerine”, assuming that these authors were the first to describe this form of muscle atrophy. According to Kazakov (1995a, 1998, 1999 and 2001), it seems that this myopathy was, however, first described by Duchenne in 1855 under the name of progressive muscular atrophy of childhood (PMACH). Later Erb (1884) and Landouzy-Dejerine (1886) described other types of myopathy resembling PMACH, referred to as juvenile shoulder-girdle (JSG) and FSHD respectively (Furukawa, 1994). PMACH was characterised as a slow progressive form that initially affected the facial, scapular and humeral muscles. Contrary to PMACH, JSG and FSHD progressed slightly quicker and initially affected the facial, scapular and peroneal muscles. These differences were initially not noted, and with time the terms PMACH of Duchenne and JSG of Erb were “absorbed” by Landouzy-Dejerine’s FSHD. This issue resulted in a relatively heated and pointless discussion between Erb and Landouzy-Dejerine on the priority of describing FSHD. This chapter deals with both clinical and genetic aspects of FSHD.

2. MUSCULAR DYSTROPHY

Muscular dystrophy (MD) is a broad term describing a diverse group of inherited disorders characterised by progressive muscle wasting and weakness. On a histological level, a large degree of muscle fibre size variation is observed, accompanied by muscle necrosis and increased amounts of fat and connective tissue (Emery, 2000). Classification criteria for the different muscular dystrophies are based on the modes of inheritance, age of onset, muscle group involvement and overall progression of the disorder. Wilhelm Erb, a well-known German neurologist, coined these disorders *Dystrophia muscularis progressiva*

(*Progressive muscular dystrophy*) at the turn of the 19th century. However, the first detailed description of a muscle related disorder was published in 1852 by an English physician named Edward Meryon. The myopathy described in that report is today referred to as Duchenne MD (Emery, 2000). Several additional dystrophies have subsequently been described and will be discussed in the following sections.

2.1 DIFFERENT TYPES OF MUSCULAR DYSTROPHY

To date, more than 30 types of MD have been recognised (Emery, 2000). The greater part of these, however, are very rare and their existences a matter of debate. Six main types of MD have been characterised, each with relatively distinctive characteristics on both the clinical and molecular levels. However, it has become clear that the MDs are a far more heterogeneous group of disorders than was previously envisaged, as even within the six major groups a great degree of variability is observed (Emery, 2002a).

2.1.1 Duchenne and Becker muscular dystrophy

“On fatty degeneration of the voluntary muscles” was the title of the first description of this form of myogenic related ailment. The clinical data, which still today characterises this disorder, was presented at the Royal Medical and Chirurgical Society Meeting in 1851 by Edward Meryon who gave a detailed description of a young boy who suffered from progressive muscle wasting until his late adolescent death. Meryon also showed that the disorder displayed familial inheritance, that it predominantly affected boys, and that the sarcolemma of muscle cells showed disruption on a histological level. Recognition for the description of this disorder was, however taken away from Meryon 16 years later when Duchenne was credited with it (Emery 2002a).

Today, the disorder has been further characterised on a clinical level with boys initially struggling to run, and later being unable to climb stairs without having to use their arms and thighs to thrust them forward and upwards. In most cases patients become dependent on the use of wheelchairs by the age of 12. Some degree of mental impairment is usual with about 20% boys having intelligent quotients (IQs) less than 70 (Emery 2002b). The affected mostly tend to die in their late teens or early twenties due to respiratory and cardiac involvement (Emery, 2002a).

Becker MD displays a similar distribution and degree of muscle weakness. The disorder is, however, not as severe as that of Duchenne MD with onset occurring at the age of 12 years and older. Some degree of mental impairment is also observed and can be accompanied by limited movement as the disorder progresses.

Both Duchenne and Becker muscular dystrophies can be explained on a genetic level by defects in the dystrophin gene located on chromosome Xp21, and fall into the category of X-linked recessive disorders (Emery, 1998). Female carriers may display some degree of muscle weakness and/or dilated cardiomyopathy. In Duchenne MD, dystrophin is usually absent, whereas in Becker it is reduced in amount or abnormal in size. This explains the wide range of phenotypic expression.

2.1.2 Emery-Dreifuss muscular dystrophy

Emery-Dreifuss MD falls into both the categories of X-linked recessive and autosomal dominant inherited disorders with both forms displaying similar clinical phenotypes. The X-linked form results from mutations in a gene that encodes the nuclear membrane protein, emerin, which is absent in muscle tissue of affected patients. The autosomal dominant type is caused by a mutation in a gene that encodes for the lamin proteins A and C. The lamins form part of the inner nuclear membrane and interact with chromatin as well as several nuclear proteins, including emerin.

On a clinical level, muscle weakening and wasting is preceded by early contractures of the Achilles tendons, elbows and posterior cervical muscles. The muscles start to weaken proximally and distally at a slow rate, followed by cardiomyopathy. The cardiac involvement generally becomes evident by the age of 30 as muscle weakness progresses.

2.1.3 Limb-girdle muscular dystrophy

Limb-girdle MD affects primarily muscles around the hip and shoulder regions. Onset is often at childhood, but it can also occur later in life. This MD displays the highest level of heterogeneity of all the muscular dystrophies with regards to both its genotype and phenotype. To date, 15 different genetic loci have been identified to be associated with

this type of dystrophy, displaying differential phenotypes and modes of inheritance. Most of the limb-girdle associated dystrophies show simultaneous impairment of intellect.

Since the MD group of disorders are so heterogeneous and the fact that the limb girdle region is affected in most of the muscular dystrophies, clinical classification has been, and still is, a complex task. In the past, when diagnoses became too difficult, disorders were often, although controversially, tossed into the classification "bin" of limb-girdle type MD. Nowadays, careful medical examination accompanied by accurate genetic analyses support the classification of disorders.

2.1.4 Facioscapulohumeral muscular dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common of all the MDs and is characterised by progressive muscle wasting of the facial, shoulder girdle and upper arm muscles (Padberg, 1982). The disorder follows an autosomal dominant mode of inheritance and affects males and females equally. The genetic rearrangement that results in the clinical phenotype involves a deletion of an integral number of repeat sequences at the D4Z4 locus on chromosome 4q35. The severity of the disorder is generally said to be proportional to the size of the deletion, however, variability is observed. The study presented here focussed on FSHD, as discussed in section 2.1.

2.1.5 Oculopharyngeal muscular dystrophy

Oculopharyngeal MD is characterised at onset by weakness of the extra-ocular and upper facial muscles. Additional weakness of the neck and proximal upper limbs completes the characteristic phenotype.

An expansion of a triplet repeat on chromosome 14q11 results in malfunctioning of a poly-(A)-binding protein and is primarily involved in the transport of mRNA from the nucleus. Excess levels of mRNA within muscle nuclei of patients with this type of MD, is therefore observed.

2.1.6 Congenital muscular dystrophy

Congenital muscular dystrophies are a group of myogenic disorders that are inherited in an autosomal recessive fashion. The disorder may be present at birth or manifests within the first few months of life. A great degree of variability is again observed on both the clinical and sub-clinical levels. Several mutations have been reported in various genes expressing the proteins laminin $\alpha 2$ (merosin), laminin receptor (integrin $\alpha 7$), fukutin, selenoprotein N1 and glycosyltransferase. Some of the different forms of congenital muscular dystrophies display mental deficiency, whilst others present with cardiac impairment.

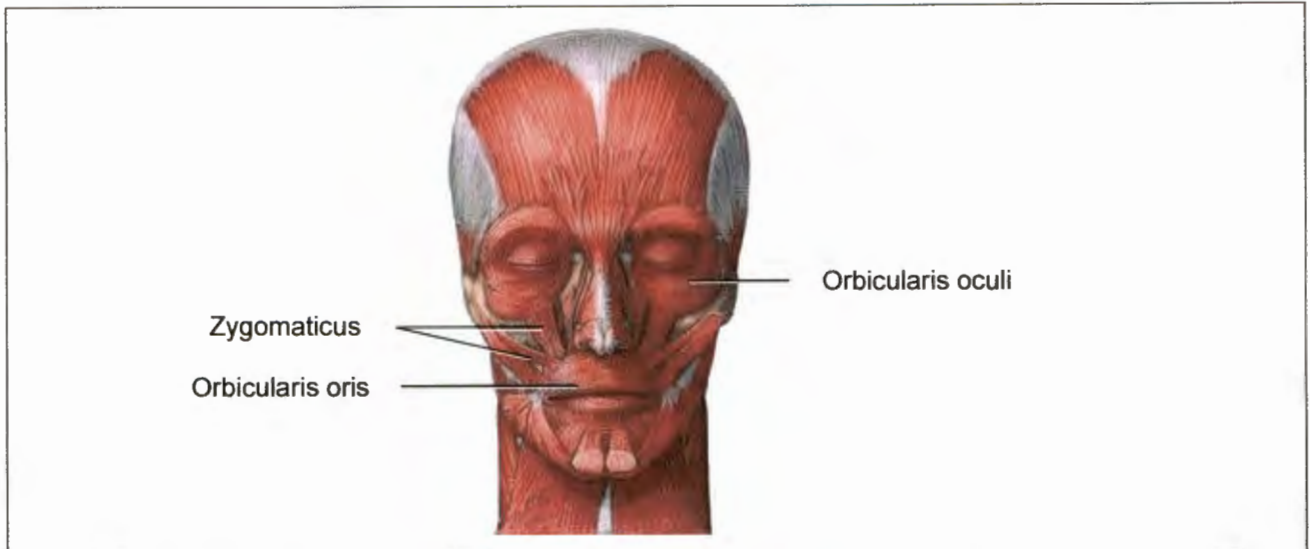
2.2 FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

FSHD is a highly variable autosomal dominant disorder that is characterised by a progressive weakness and atrophy of the facial, shoulder girdle and upper arm muscles with subsequent involvement of the trunk, pelvic girdle and thigh muscles. The life expectancy of affected FSHD patients appears to be normal. Equal incidence in males and females is observed.

2.2.1 Clinical phenotype of FSHD

Typical symptomatic presentation of FSHD is generally observed in the second decade of life. The examination initially reveals characteristic peri-oral and/or peri-orbital facial weakness. Oral weakness is due to deterioration of the orbicularis oris and zygomaticus muscles (Figure 2.1). Zygomaticus deterioration results in an inability to raise the corners of the mouth, which in turn causes a subsequent “transverse smile” (Padberg, 1982). A weakened orbicularis oris muscle of the mouth prevents the patient from retaining air under pressure and makes it difficult to whistle. The lips appear full and are often everted.

Figure 2.1: Diagrammatic representation of facial muscles primarily affected in FSHD

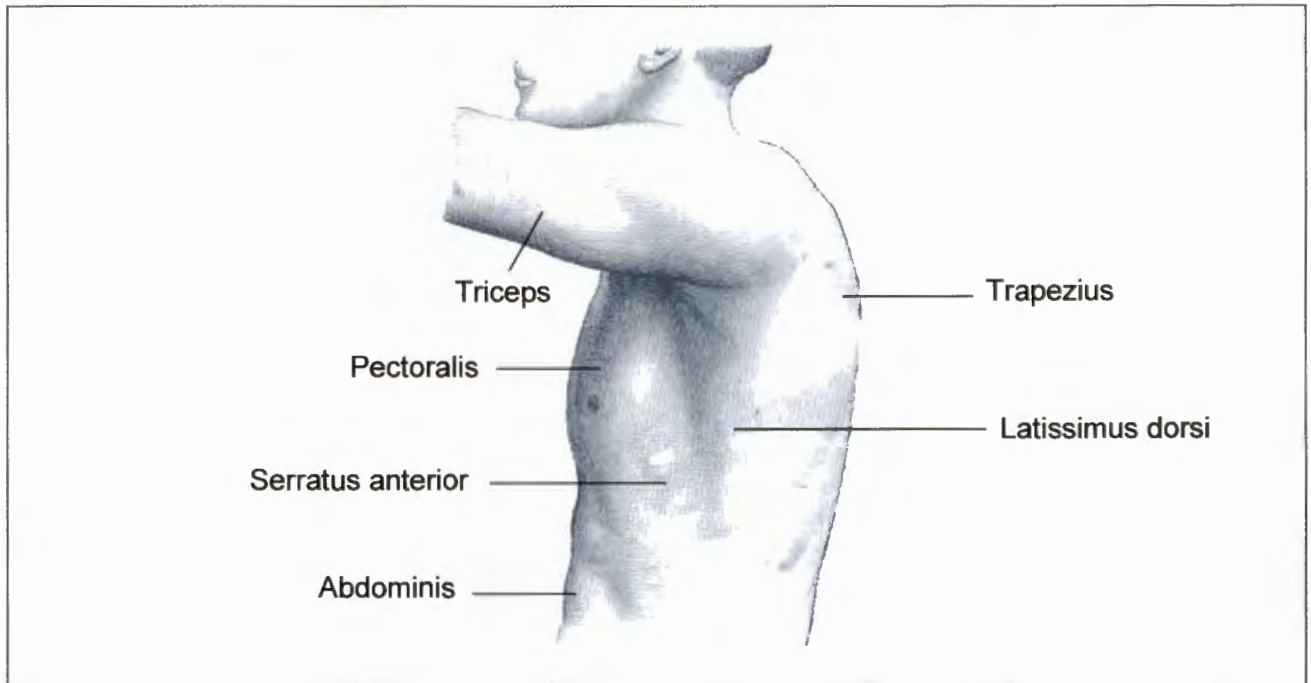


Adapted from thailabonline.com/muscle/face.htm.

The orbicularis oculi muscles, around the eyes, function to close the eyelids and aid the passage and drainage of tears. Deterioration of this muscle is less severe than the peri-orals (Padberg, 1982). However, severely affected patients tend to suffer from additional facial weakness to a considerably greater degree. The eyes may seem prominent, and patients often appear to sleep with their eyes partially open. Surrounding facial muscles may also become affected at this stage, resulting in an unlined forehead and a “Möbius-like” expressionless face.

Apparent muscle wasting and weakening of the shoulder girdle and upper-arm generally follows, revealing the characteristic FSHD phenotype. The primary muscles affected include the trapezius, rhomboids and serratus anterior (Figure 2.2). Surrounding and supportive muscles to the abovementioned may also become affected as the disorder progresses. Collectively, these muscles share the function of fixating and stabilising of the scapula to the torso. When these muscles become affected and start to weaken, the result is scapulae alatae, alternatively referred to as winging of the scapulae, as patients attempt to move their arms outward or forward. This change in the position of the scapulae contributes to the development of a characteristic posture with the shoulders thrust forward. Atrophy of the pectoral muscles as well as the biceps and triceps gives the appearance of flattened chest walls and extremely thin upper-arms respectively (Padberg, 1982; Padberg *et al.*, 1997).

Figure 2.2: Surface anatomy of the thorax illustrating muscles that are primarily affected in patients with FSHD



Adapted from Henry Gray's *Anatomy of the Human Body*, 2000.

Muscle atrophy in patients with FSHD is progressive in nature and regression of symptoms and signs does not occur. The rate and severity of progression varies from patient to patient. As a general rule, atrophy does tend to progress at a higher rate in individuals with earlier ages of onset (as will be discussed in section 2.2.1.1.2). Progression of the disorder usually involves the abdominal muscles and proximal lower limbs to an extent that wheelchairs may be required in 10-20% of cases (Lunt and Harper, 1991). Pelvic girdle and upper arm weakness may occur at any time after the onset of shoulder girdle weakness (Padberg *et al.*, 1997).

2.2.1.1 Heterogeneity of FSHD

This wide range of phenotypic variability can be demonstrated not only between families, but also to a large extent within families. This variability is clearly visible when comparing patients with signs of asymptomatic scapular girdle or facial weakness in the 7th decade, to cases with severe progressive proximal and distal upper and lower limb involvement together with “Möbius-like” expressionless faces by early childhood (Jardine *et al.*, 1994; Lunt, 2000).

2.2.1.1.1 Sex influence

There has been much speculation over the years with regards to sex influence on the FSHD phenotype. Padberg *et al.* (1995b) obtained data, although not statistically significant, indicating that there are twice as many asymptomatic female carriers of the disorder than males. The most recent figures (Zatz *et al.*, 1998) indicate that not only are a greater proportion of males affected with the disorder, but that there is a higher percentage of penetrance of the rearranged locus in males i.e. 95% for males and 69% for females. The fact that previous observations were based on clinical and pedigree analyses and not on subsequently improved DNA strategies, adds increased credibility and reliability to the observations of Zatz and her colleagues.

2.2.1.1.2 Infantile FSHD

Early onset or infantile FSHD is a peculiar observation that is said to contribute to the clinical heterogeneity of FSHD. Several cases of such nature have to date been reported. Infantile FSHD is characterised by similar myopathogenesis as typical FSHD, except that onset occurs within the first decade of life and that it is generally accompanied by a higher degree of variability and severity to the extent that extra-muscular involvement (see section 2.2.1.1.3) may be present (Brouwer *et al.*, 1994). Due to extra-muscular involvement in addition to the early onset phenotype, a separate form of FSHD was previously suggested. However, with the developments in genetic testing, these patients were proven to harbour similar DNA rearrangements at the D4Z4 locus (section 2.2.2.1), the only notable difference being that of a slightly larger deletion. In 1998, Funakoshi *et al.* reported deletion fragments of 10 – 11 kb in length in nine out of twenty infantile patients investigated. Small deletion fragments of 13 kb and 15 kb in early onset FSHD patients were also reported by Okinaga *et al.* (1997). It became clear with time that early onset FSHD forms part of the broad spectrum of the FSHD phenotype.

2.2.1.1.3 Extra-muscular involvement

In a significant number of patients, extra-muscular symptoms such as retinal vasculopathy and sensorineural deafness have also been observed to form part of the clinical spectrum (Padberg *et al.*, 1995a). Brouwer *et al.* (1991) reported that they undertook a study to determine how often hearing loss accompanied a large population of patients affected with

autosomal dominant FSHD. Seventy-two healthy control family members and 56 FSHD positive members from nine families were investigated. Diagnoses were based on a clinical level and on linkage analysis with loci associated with the disorder. Ages ranged from 14 to 77 years, with approximately equal numbers of males and females. The results indicated that a difference in hearing levels was significantly greater in FSHD patients than in controls. The severity of hearing loss varied between subjects at any age and tended to be progressive with the advancement of the disorder. There was, however, no clear relationship observed with either hearing loss or age and severity of the phenotype in the FSHD patients investigated. Hence it was concluded that a change in hearing function formed part of the FSHD phenotype and may lead to severe hearing loss in some patients.

Retinal vascular abnormalities have also been reported to be associated with FSHD. Fitzsimons *et al.* (1987) investigated the relationship of FSHD with retinopathy in 75 FSHD individuals. In four of the nine families investigated, at least three or more individuals showed evidence of retinal vascular disease, and in the remaining five families at least one member displayed an abnormal angiogram. In total, a statistically significant 57 out of 75 individuals (75%) presented with retinopathy associated FSHD. However, no correlation between the severity of FSHD and the degree of retinal abnormality was observed. It was hence concluded from the Fitzsimons study, that retinal vascular disease appeared to be an integral part of FSHD.

Although little attention has been paid to intellectual function in patients affected with FSHD, such incidences have been reported. Padberg *et al.* (1995a) reported four cases of FSHD diagnosed males with mental impairment in a survey of 256 patients. Three suffered from early onset FSHD and the fourth from a severe case of retinal vascular abnormality.

Since cardiac involvement has been demonstrated to be prevalent in other forms of MD i.e. Duchenne and Becker, Limb girdle and Emery-Dreifuss, an association to FSHD was also investigated. Yamamoto *et al.* (1988), Stevenson *et al.* (1990) and Faustman *et al.* (1996) had all reported cardiac involvement associated with FSHD. Even though varied prevalences were reported, the incidences were significant. Faustman *et al.* (1996) illustrated that only those FSHD patients whose phenotype had been confirmed on the genetic level, showed signs of cardiac defects and/or dysfunctions. It was suggested that, even though there is speculation regarding the role of cardiac involvement in the clinical

phenotype of FSHD, patients should be kept under close supervision prior to, and during, any kind of anaesthetic related surgery or examination.

2.2.1.2 Clinical management of FSHD

The primary symptom of patients affected with FSHD is that of loss of total shoulder abduction owing to absence of scapular stabilisation to the thoracic wall. When the patient tries to perform activities above shoulder level it results in winging of the scapulae away from the chest wall and subsequent limited abduction. Patients generally complain about difficulty with raising their arms and performing any form of tasks above shoulder level. An increase in shoulder abduction can be made evident to the patient if the examiner holds the scapula firmly between the hands and the posterior chest wall, and then asks the patient to abduct the arm as much as possible. This effect can be surgically accomplished by either ligamentous stabilisation or bony fusion of the scapulae to the ribs. The procedure is referred to as scapulothoracic arthrodesis and has previously been described by Letournel *et al.* (1990) and Bunch and Siegel (1993). Since the lifespan of patients affected with FSHD is not limited, the procedure may be both viable and advantageous to the individual, as it will allow for increased ability in performing daily chores.

Post-operatively, all the results of scapular fixation to ribs have been reported to improve forward flexion and abduction to at least ninety degrees in patients, with minor complications occurring (Letournel *et al.*, 1990). Patients reported increased stability and strength while carrying or lifting objects as well as improved ability to perform the simple tasks of daily living, such as dressing, combing hair and reaching for objects above shoulder level. Andrews *et al.* (1998) investigated the long term effectivity of the procedure. Over a mean follow-up period of 73.2 months, nine out of ten shoulders that underwent scapulothoracic arthrodesis showed no loss of the initially regained movement.

Orthotic treatment using a tethered sling or brace has also previously been prescribed to patients with FSHD who would prefer the route of prosthetics rather than surgery. Several modalities have been described, such as scapular pads, a spica brace, a scapula alata brace, canvas shoulder braces with steel bars at each side of the back functioning as a scapular orthosis and a serratus anterior restraining brace (Alsancak *et al.*, 2000).

Unfortunately, even though improved arm extension and movement was achieved, these braces often tended to cause discomfort among patients.

Alsancak *et al.* (2000) developed a novel brace that was designed as a jacket and covers the thorax and both shoulders of the patient. The jacket was made from polyethylene and had polyform padding in selected regions to ensure as much comfort as possible. Anterior openings enable easy dressing of the jacket. Two scapular cups were designed to allow for scapulothoracic fixation. When compared to previous orthoses, the Alsancak brace showed to be superior in terms of comfort and active shoulder range of motion. Patients also displayed increased relative muscle strength of the thorax and subsequently were able to use their arms and upper body for prolonged periods with the orthosis.

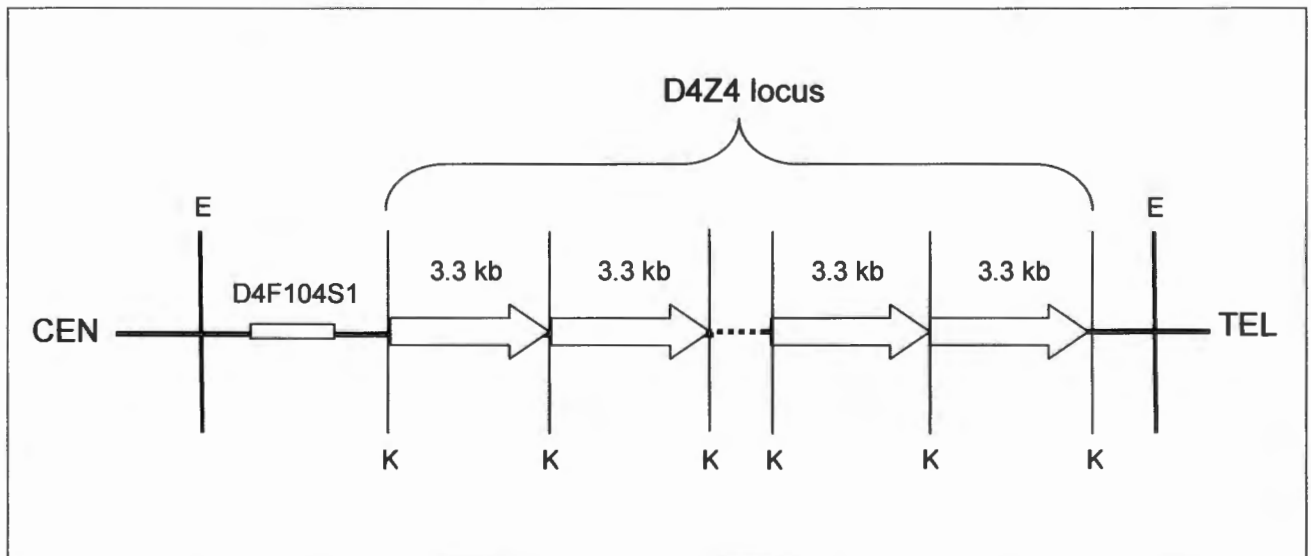
2.2.2 Genetic aspects of FSHD

The inheritance pattern of FSHD is according to an autosomal dominant Mendelian mode with a penetrance of approximately 95% by the age of 20 years (Lunt *et al.*, 1989). Advances in molecular genetics have led to the mapping of various neurologically related disorders, such as Duchenne, Becker and the other previously discussed MDs (section 2.1). Several linkage studies have been performed to identify the FSHD locus. A genome-wide scan with polymorphic microsatellite markers on distinct affected and non-affected members of well-characterised FSHD families was performed by Wijmenga *et al.* (1990). One of these microsatellite markers, Mfd22, displayed linkage to the putative FSHD locus, D4S171, on chromosome 4q. The FSHD locus was subsequently demonstrated to be even more closely linked to locus D4S139, which was mapped to the distal portion of the long arm of chromosome 4 via *in situ* hybridisation (Wijmenga *et al.*, 1991; Matthews *et al.*, 1992; Mills *et al.*, 1992). This firmly established the location of the FSHD associated locus to the chromosomal region 4q35. Polymorphic markers (D4F35S1, D4F104S1 and D4Z4) between D4S139 and the 4q35 region were utilised in the further characterisation of this interval. These genetic markers were ordered via various other mapping methodologies which demonstrated that D4F104S1 and D4Z4 appeared to be directly associated with the FSHD locus (Wijmenga *et al.*, 1993a). Since then, additional markers have become available, forming a linkage group of several polymorphic loci in the area of this FSHD locus (Upadhyaya *et al.*, 1993; Weiffenbach *et al.*, 1994; Winokur *et al.*, 1993; Wijmenga *et al.*, 1995).

2.2.2.1 The D4Z4 locus on chromosome 4q35

FSHD is caused by a mutation mechanism that is currently still unique amongst genetic conditions in the human genome. The DNA mutation is known, but there is to date no gene or genes identified to be directly causative of FSHD (Lunt, 2000; Altherr *et al.*, 1995; Forrester *et al.*, 1998). The genetic defect causing FSHD is a deletion of an integral number of 3.3 kilobase pair (kb) repeat units within locus D4Z4, which are located at the 4q35 sub-telomeric chromosome region (Figure 2.3). The rearranged locus can be detected and analysed by *Eco* RI restriction enzyme digestion of genomic DNA (gDNA) and hybridisation with probe p13E-11, which identifies the locus D4F104S1. Each of the 3.3 kb repeats are flanked by a *Kpn* I restriction site and the repeats have identical restriction patterns (Lee *et al.*, 1995). Unaffected individuals have ten to ninety-six tandem repeat copies of these 3.3 kb units, whereas affected individuals display as little as one to ten copies (Van Deutekom *et al.*, 1993; Wijmenga *et al.*, 1993b). Thus, in samples from FSHD patients, the *Eco* RI fragments generated are smaller than 36 kb, while the fragments generated in samples from unaffected individuals vary from 36 to 300 kb (Wijmenga *et al.*, 1992).

Figure 2.3: Diagrammatic representation of the sub-telomeric region of chromosome 4q35



K = *Kpn* I restriction enzyme site, E = *Eco* RI restriction enzyme site, TEL = telomere, CEN = centromere. Adapted from Fisher and Upadhyaya, 1997

The repeat arrays of D4Z4 reveal a base composition that is 71% GC and restriction analysis indicated that the locus is part of a CpG island (Wijmenga *et al.*, 1993b). This characteristic is common to heterochromatic regions of DNA and renders the locus

sensitive to DNA methylation (Bird, 1986). Yeast artificial chromosome (YAC) cloning and a detailed restriction map revealed exclusive rare cutting sites that cannot be detected by enzymes sensitive to methylation, hence indicating that these sites are methylated (Wijmenga *et al.*, 1993a).

A double homeobox domain sequence element has been identified in each D4Z4 repeat. This element may be associated with a transcript as it is situated within a putative open reading frame of 405 bp (Hewitt *et al.*, 1994). Homeobox domains of proteins are known to bind specific DNA sequences and generally function to activate or repress transcription of target genes, which are primarily involved in developmental processes (McGinnis and Krumlauf, 1992).

Unfortunately, the transcriptional activity of D4Z4 has been demonstrated to be minimal. Hence, a position effect variegation (PEV) model has been proposed for the manifestation of the FSHD phenotype. This model proposes that the deletion of a critical number of D4Z4 repeat units influences the expression of nearby located genes. PEV is traditionally believed to be caused by heterochromatisation, and genes juxtaposed next to such regions as a result of DNA rearrangements, often exhibit variegated expression (Winokur *et al.*, 1994). Heterochromatisation may also cause a structural change in chromatin condensation and ultimately result in transcriptional silencing of a region (Bedell *et al.*, 1996; Moehrle and Paro, 1994). The D4Z4 repeat array may, in fact, represent a buffer zone between telomeric heterochromatin and euchromatin. Deletion of a critical number of the D4Z4 array units may then result in expression alteration of genes located in the proximal vicinity (see section 2.2.2.6). As a consequence, a gene associated with FSHD may be adversely affected and cause, or contribute to, the FSHD phenotype.

2.2.2.2 Chromosome 10q26

Southern blot analysis revealed that probe p13E-11 cross-hybridises to several other regions in the human genome. Subsequent fluorescent *in situ* hybridisation (FISH) analyses of 4q identified homologous sequences on all the acrocentric chromosomes, 10q and the pericentric regions of chromosomes 1, 3, 9, 10 and Y (Hewitt *et al.*, 1994, Winokur *et al.*, 1994, Altherr *et al.*, 1995).

The most significant of the reported cross hybridisations is to fragments in strikingly similar size ranges and patterns to those originating from D4Z4. Wijmenga *et al.* (1993b) illustrated that, of the two pairs of polymorphic alleles observed via Southern analyses, one was 4q35 specific and the other not linked to this region. Since these fragments were of similar size, they sometimes overlapped upon Southern blot analysis. The detection of additional fragments with probe p13E-11 severely complicated FSHD diagnosis, leading to their conclusion that this probe did not detect FSHD rearrangements with 100% sensitivity.

Deidda *et al.* (1995) observed a small fragment of 13 kb, not related to the disorder, segregating in one of their FSHD families along with a 23 kb fragment from chromosome 4q35. This non-4q35 fragment was cloned and with subsequent haplotype analysis and *in situ* hybridisation, it was demonstrated that this fragment originated from chromosome 10q26. *Eco* RI fragments originating from chromosome 10q26 may be up to 300 kb in size, and may even be less than 36 kb. This region is, however, not associated with FSHD and hence deletion fragments at this locus are non-pathogenic (Bakker *et al.*, 1995). Consequently, shortened fragments from 10q, which occur in approximately 10% of the human population, may incorrectly be assigned to 4q35, resulting in FSHD misdiagnosis (Bakker *et al.*, 1996). It became clear that it was essential to find means of distinguishing the 4-type and 10-type fragments from each other in order to obtain accurate diagnoses.

Upon comparative sequence analysis of the 4-type and 10-type repeat sequences, a sequence homology of 98% to 100% was observed (Cacurri *et al.* 1998). More recently, Van Geel *et al.* (2002) reported analyses reflecting extended sequence homology. One of the aims of their study was to provide reasoning for the lack of association of 10q with the FSHD phenotype based on the chromatin organisation. Contrary to their hypotheses, the results indicated that sequence homology extends 42 kb proximally, as well as distally to include the telomere. Thus, a difference in telomeric chromatin organisation is an unlikely explanation for the lack of association between the short 10q 3.3 kb repeat arrays and the FSHD phenotype.

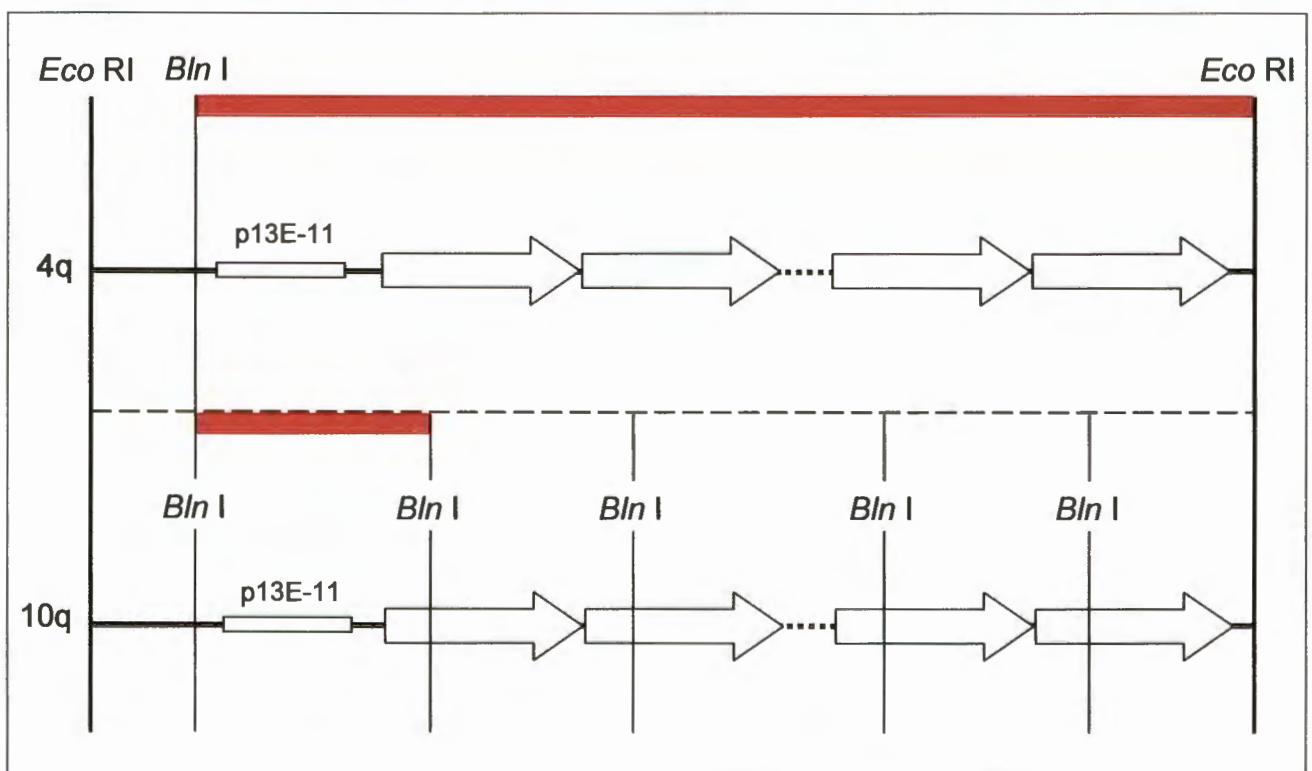
2.2.2.3 Distinguishing 4-type and 10-type fragments

Comparative sequence analysis of the 3.3 kb repeated units derived from chromosomes 4q35 and 10q26 revealed the presence of chromosome specific restriction enzyme sites

within the respective repeat sequences. A *Bln* I restriction site was initially described to be present within each of the repeat units originating from chromosome 10q26 (Deidda *et al.*, 1996; Cacurri *et al.*, 1998). Later, Lemmers *et al.* (2001) reported findings of *Xap* I restriction enzyme sites within each of the repeats from chromosome 4q35. Together, these findings enabled optimised FSHD diagnosis.

Southern blot analysis with p13E-11 on *Eco* RI - *Bln* I double digested genomic DNA enabled accurate differentiation of the chromosomal *Eco* RI fragments, since short 10q26 specific *Eco* RI segments are eliminated by *Bln* I digestion (Figure 2.4). Since there is also a digestion site for *Bln* I approximately 3 kb distal from the first *Eco* RI site, all chromosome 4 specific fragments digested with *Bln* I and *Eco* RI will therefore be visualised as shorter when compared to those digested with *Eco* RI alone. The identification of the *Bln* I site brought about considerable improvement in the reliability of FSHD diagnosis with improved sensitivity of 92% and specificity of 99% using conventional gel electrophoresis (Van der Maarel *et al.*, 1999). When pulsed field gel electrophoresis (PFGE) is utilised the sensitivity and specificity can be further improved to values of 96.5% and >99% respectively.

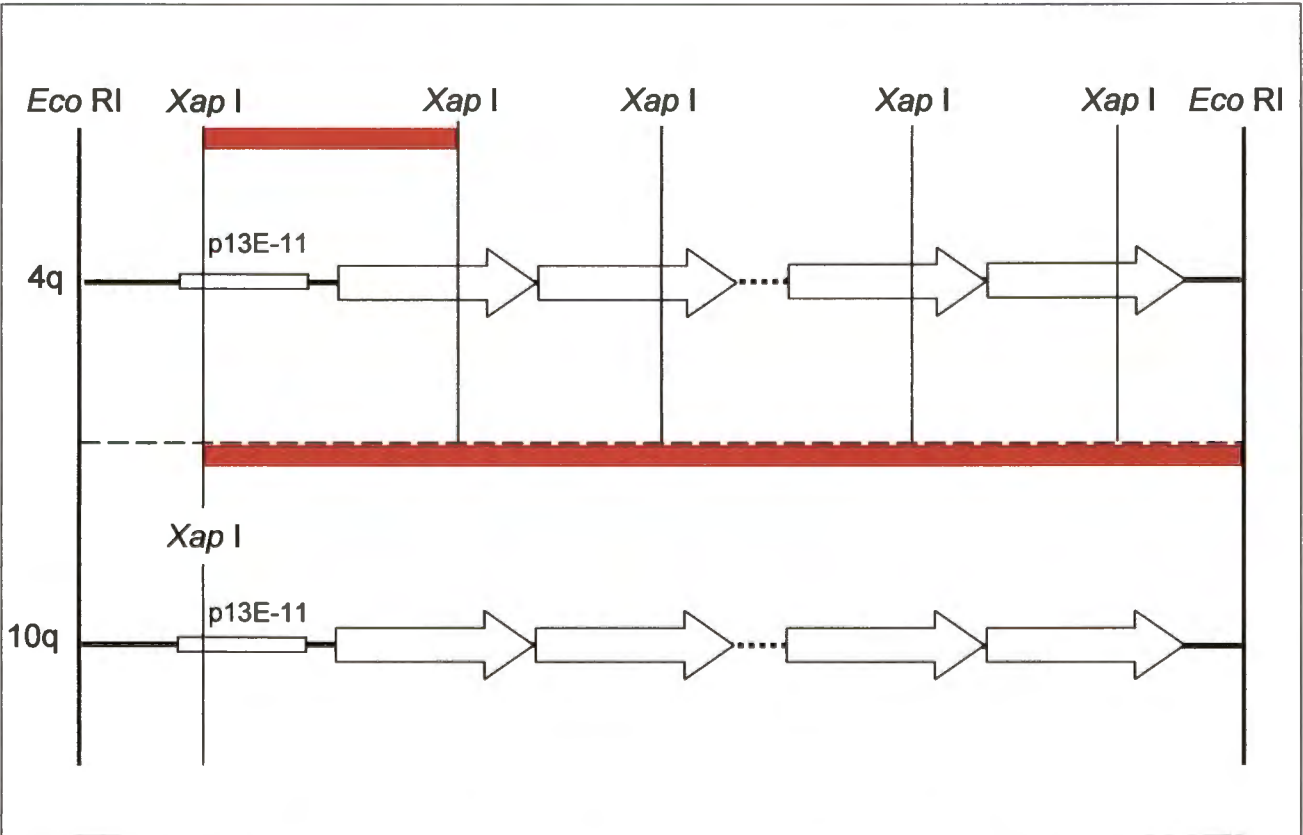
Figure 2.4: Fragments generated subsequent to *Eco* RI – *Bln* I double digestion



The arrays are located within a digested *Eco* RI fragment. The restriction enzyme *Bln* I digests only the 10q derived repeat sequences as indicated on the chromosome below the dotted axis line. The fragments generated from the double digestion that are detected by p13E-11 are indicated in red. Adapted from Deidda *et al.*, 1996.

Until recently the *Eco* RI-*Bln* I double digestion was the only means of discriminating 4-type repeats from those originating from chromosome 10. However, sequence characterisation of chromosome 4-type repeats has revealed additional differences that can be exploited to complement the *Eco* RI - *Bln* I diagnostic approach. Similar to the repeats from chromosome 10, a characteristic *Xap* I restriction enzyme site has been identified in each of the repeat units originating from chromosome 4q (Lemmers *et al.*, 2001). Comparative sequence analyses of the two respective repeat sequences displays a consistent difference between each of the derived units. A transition of G to C at position 7515 (GenBank accession number AF117653) results in the presence of the *Xap* I restriction enzyme site in each of the repeat units arising from chromosome 4. Therefore, in a similar fashion to the *Eco* RI - *Bln* I test, a double digestion reaction with *Eco* RI and *Xap* I will result in the subsequent elimination of 4-type fragments thus enabling discrimination between fragments from 4q and 10q (Figure 2.5).

Figure 2.5: Fragments generated subsequent to *Eco* RI – *Xap* I double digestion



The arrays are located within a digested *Eco* RI fragment. The restriction enzyme *Xap* I digests only the 4q derived fragments as indicated on the chromosome above the dotted axis line. The fragments generated from the double digestion that are detected by p13E-11 are indicated in red. Adapted from Lemmers *et al.*, 2001.

A *Xap* I digestion site is also present in the D4F104S1 locus, to which probe p13E-11 hybridises, resulting in a slightly weakened hybridisation signal. However, the signal

intensities are still sufficient for diagnostic purposes (Lemmers *et al.*, 2001). Since the 10-type fragments are completely resistant to *Xap* I and eliminated by an *Eco* RI-*Bln* I double digestion, the additional double digestion reaction with *Xap* I and *Eco* RI will not only complement and enhance the existing diagnostic protocols, but also allow for complete allele elucidation.

2.2.2.4 Translocation events between chromosomes 4q35 and 10q26

Differential restriction analyses utilising *Eco* RI and *Bln* I indicated the presence of chromosome 4q35 specific 3.3 kb repeat arrays on chromosome 10q26 and *vice versa* (Van Deutekom *et al.*, 1996a). Therefore, several affected as well as unaffected individuals appeared to be monosomic or trisomic, or even nullosomic or quatosomic for the 4q35-linked p13E-11 fragment. These findings suggest the occurrence of sub-telomeric interchromosomal repeat exchange events between these highly homologous sequences. The frequency of sub-telomeric translocations between 4q35 and 10q26 in the Dutch population is at least 20% (Van Deutekom *et al.*, 1996a, Van der Maarel *et al.*, 1999). Matsumura *et al.* (2002) undertook a study to determine the frequency of 4q - 10q translocations amongst different ethnic populations. They examined 153 Japanese, 124 Korean and 114 Chinese healthy individuals as well as 56 Japanese patients affected with FSHD. Table 2.1 illustrates the frequency of translocation events that were recorded in this study as well as frequencies that were previously obtained from studies performed in the Dutch population. The frequencies that are indicated are combined scores of the translocation events of 4-type repeats to chromosome 10 and 10-type repeats to chromosome 4. It is clear that there were no significant differences observed between patients affected with FSHD and control individuals. Interestingly, little variation in frequency was observed when comparing each of the Eastern populations and even more importantly, these values paralleled with those obtained for the Dutch population as reported by Van Deutekom *et al.* (1996a) and Van Overveld *et al.* (2000). It is evident that from the populations genotyped to date, a relatively consistent translocation frequency has been recorded. Via characterisation of the South African FSHD population with regards to subtelomeric plasticity of the chromosomal 4q and 10q regions, it will be elucidated whether the previously recorded frequency of translocation also pertains to this population. Since this study investigates the Caucasian population, which has strong Dutch and European ancestry, it was postulated that similar translocation frequencies would be obtained.

It has been suggested that due to the high degree of homology between the telomeric regions, it is possible that a type of interchromosomal “cross-talk” may exist between these regions (Van Overveld *et al.*, 2000). These sub-telomeric rearrangements generated a novel interchromosomal polymorphism, which negatively affects the specificity and sensitivity of the differential DNA restriction analysis for FSHD diagnosis. The mechanism behind the sub-telomeric exchanges remains to be elucidated. Similar translocation events are relatively common and have been reported to play a causal role in several other disorders, such as idiopathic mental retardation syndrome (Flint *et al.*, 1996) and Miller-Dieker syndrome (Kuwano *et al.*, 1991). The interchromosomal exchanges do indeed have implications for the aetiology of FSHD. As discussed earlier, it is only a deleted 4q35-linked *Eco* RI fragment that results in FSHD. Therefore, a *de novo* exchange resulting in a shortened fragment from chromosome 4q, but containing repeat units that were originally from 10q26, will also cause FSHD.

Table 2.1: Translocation frequencies of four different populations

Population Translocation Frequencies				
	Dutch	Japanese	Chinese	Korean
FSHD	0.200 ^(a)	0.321 ^(c)	—	—
Control	0.210 ^(b)	0.275 ^(c)	0.193 ^(c)	0.298 ^(c)
Average	0.205	0.298	0.193	0.298
Mean	0.2485			

(a) Van Deutekom *et al.*, 1996a; (b) Van Overveld *et al.*, 2000; (c) Matsumura *et al.*, 2002.

Further sub-telomeric instability has also been observed in that allelic 3.3 kb repeat arrays may have a hybrid structure composed of chromosome 4 and 10 derived repeat units, suggesting that intra-chromosomal cross-talk may also exist (Lemmers *et al.*, 1998). Lemmers and his colleagues hybridised DNA of apparent monosomic and trisomic individuals with probe 9B6A, which recognises each of the repeats within the D4Z4 locus, in order to study the repeat units themselves. Their results indicated that, in certain cases of FSHD, a more complex hybridisation pattern consistent with a hybrid structure of the repeat units at this locus was observed. It is not known whether these hybrid repeat loci disturb transcribed sequences within the repeat unit itself, and it can therefore not be ruled out as a factor that may contribute to the clinical phenotype of FSHD.

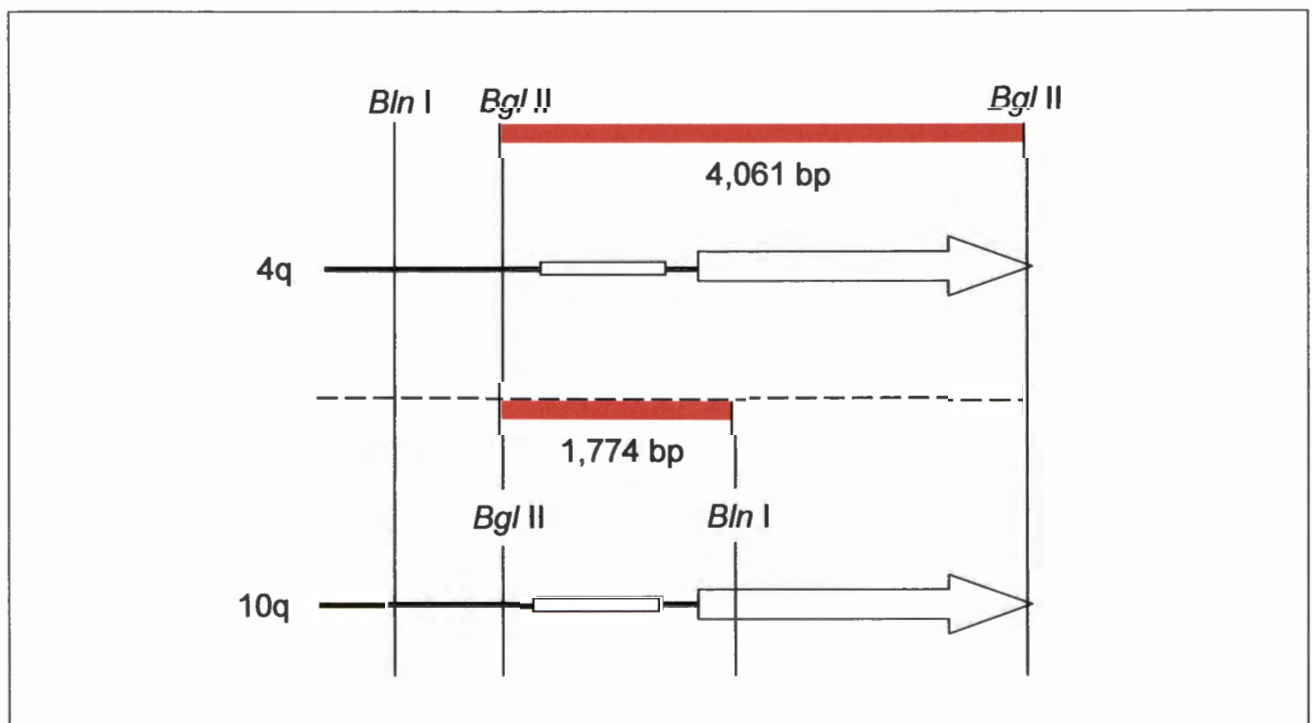
2.2.2.5 *Bgl* II – *Bln* I dosage test

There are unfortunately several factors that complicate accurate molecular analyses and diagnoses of FSHD patients (Lemmers *et al.*, 1998). Consequently, specificity and sensitivity of DNA analyses are compromised. The following are major contributors:

- Probe p13E-11 cross hybridising with several different loci in the human genome.
- Deletions of locus D4F104S1, to which p13E-11 hybridises.
- Translocation events occurring between chromosomes 4q and 10q.

In 1999 Van der Maarel *et al.* reported an easy and reliable Southern blot method to address the above mentioned issues and further elucidate DNA rearrangements related to FSHD. The method described is referred to as the *Bgl* II-*Bln* I dosage test and enhances specificity and sensitivity of the conventional FSHD diagnostic test. The test exploits only the first repeat sequence of each array. It uses the *Bln* I polymorphism within the first D4Z4 repeat unit and *Bgl* II instead of *Eco* RI to obtain a shortened fragment. Figure 2.6 represents a schematic overview of the generated fragments and respective sizes by the *Bgl* II-*Bln* I dosage test.

Figure 2.6: Schematic overview of the fragments generated via the *Bgl* II - *Bln* I dosage test



Adapted from Van der Maarel *et al.* (1999).

It is evident from Figure 2.6 that a chromosome 4 derived fragment of 4,061 bp will be released after the double digestion, and that the characteristic *Bln* I site within each of the chromosome 10 repeat arrays will result in a shortened 1,771 bp fragment. The signal intensities generated from the chromosome 4 and chromosome 10 fragments enables determination of the fragment ratios. Therefore, if a translocation event occurred, the skewed ratios generated from the dosage test will enable its recognition via a visual inspection (Van der Maarel *et al.*, 1999).

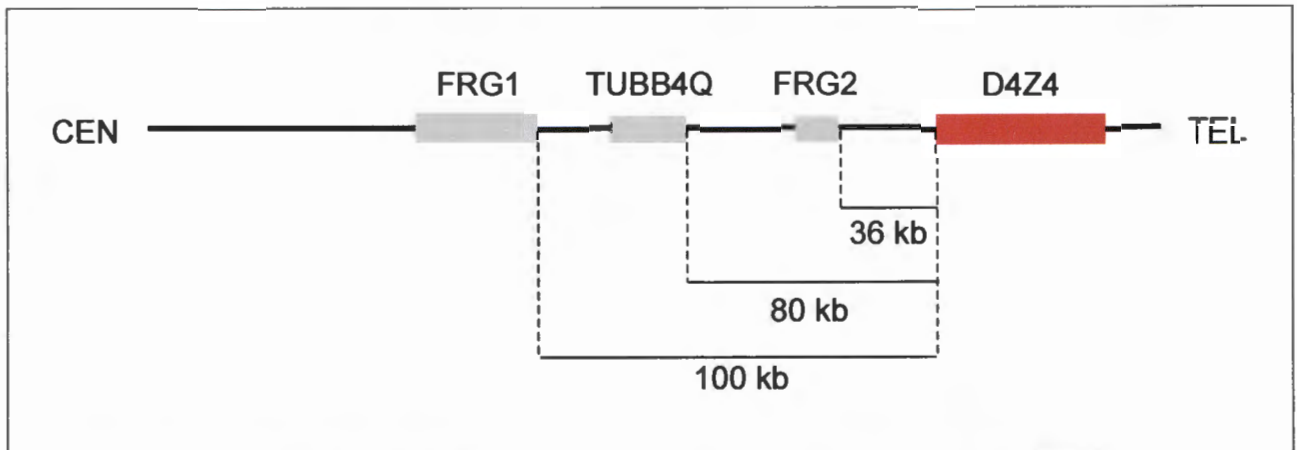
Since the *Bgl* II - *Bln* I dosage test can be performed with relative ease and can be used to pinpoint the occurrence of a translocation event, it has become a widely used and internationally accepted procedure. It is for this reason that this method was selected to investigate the occurrence of translocation events in the South African population.

2.2.2.6 Candidate genes linked to chromosome 4q35

FSHD candidate gene analysis was initially performed on the sequences that are causally associated with this disorder. However, minimal transcriptional activity of the D4Z4 locus has been demonstrated. FSHD is caused exclusively by a shortened 3.3 kb repeat array on chromosome 4q35, regardless of its origin and repeat composition, which may be altered due to inter- and intra-chromosomal DNA rearrangements. The fact that the repeated units most likely do not constitute any part of the FSHD gene (section 2.2.2.1) suggests that the PEV hypothesis is the most likely mechanism for FSHD.

The identification of associated genes in the 4q35 region was a difficult undertaking (Van Geel *et al.*, 2001). The main reasons being due to the large amount of homologous regions to the FSHD locus residing elsewhere in the genome, the high density of repeats, and limited number of genes in the region. DNA sequencing allowed for a complete chromosomal scan of the entire region for genes and other sequence elements that may be associated with FSHD. Figure 2.7 illustrates candidate genes proximal to the D4Z4 locus that have been reported to date and may be linked with the FSHD phenotype. The three genes illustrated will be discussed in the remainder of this section.

Figure 2.7: Diagrammatic representation of possible candidate genes in the 4q35 region



The diagram illustrates the candidate genes within the 4q35 region in relation to the D4Z4 repeat sequences. CEN = centromere, TEL = telomere, FRG1 = FSHD region gene 1, TUBB4Q = Human β -tubulin Q4, FRG2 = FSHD region gene 2. Adapted from Van Geel *et al.*, 2001.

2.2.2.6.1 FSHD region gene 1 (FRG1)

FRG1 was the first functional gene identified within the FSHD region on chromosome 4q35 (Van Deutekom *et al.*, 1996b). PEV may affect genes over a region of several hundred kilobases. It is for this reason that gene identification methods were adopted to analyse up to approximately 150 kb proximal to the 3.3 kb repeat array. Identification of FRG1 was achieved in two stages. A DNA fragment associated with a CpG island located 100 kb from D4Z4 was used as a probe to screen a human skeletal muscle cDNA library. Positive clones were identified from these experiments along with subsequent recovery of full-length cDNA (Van Deutekom *et al.*, 1996b). Secondly cosmids were subjected to an exon trapping procedure, which resulted in identification of five of the nine exons of FRG1. The complete cDNA sequence of this novel human gene was compiled, spanning 1,042 bp and encoding a protein of 258 amino acid residues. Several pseudogenes were also identified, each originating from a different chromosome. The FRG1 protein was found to be highly expressed in several other human tissues, for example the brain, placenta and lymphocytes. The function of the identified gene is still unknown. The FRG1 protein localises in the nucleolus and it has been postulated to play a fundamental role in RNA processing (Van Koningsbruggen *et al.*, 2000).

To investigate the possibility of a PEV mechanism in this region, which could causally link the FRG1 gene to FSHD, allele-specific transcript levels of FRG1 in patients and controls were examined by RNA-based single-strand conformation polymorphism (SSCP) analysis

(Van Deutekom *et al.*, 1996b). These results would confirm whether transcriptional repression occurs in FSHD patients or not. A polymorphic fragment contained within the first exon of FRG1 was amplified from reverse transcribed RNA from lymphocytes and muscle biopsies. In multiple experiments no significant transcription repression was detected in the reverse transcribed RNA isolated from the lymphocyte and muscle tissues of FSHD patients. These results suggested that an FSHD associated deletion of repeated units does not influence the transcript levels of FRG1 in at least these tissues (Van Deutekom *et al.*, 1996b). Further studies, based on the transcription levels in more specific cell types, would be needed in order to provide sufficient evidence for PEV-induced differential expression of FRG1.

2.2.2.6.2 Human beta-tubulin gene (TUBB4Q)

Using the same method of exon trapping that identified the FRG1 gene, a sequence with high homology to beta-tubulin gene sequences was identified (Van Geel *et al.*, 2000). One of the exon trapped clones, pET277, mapped approximately 80 kb proximal to the D4Z4 locus and was designated TUBB4Q. Sequence analysis revealed a high degree of homology (87%) to a β 2-tubulin sequence. This high level of homology can be expected as the β -tubulins are part of vital processes in the cell, which puts a structural and functional constraint on evolutionary amino acid changes. The predicted mRNA from the genomic sequence contains an open reading frame (ORF) of 1,302 bp, encoding a putative protein of 434 amino acids.

This novel tubulin gene contains intronic sequences similar to that of functional β -tubulin family members. There are, however, amino acid substitutions in protein domains that are highly conserved among β -tubulins. These substitutions along with high allelic sequence variability found in TUBB4Q, suggests that this gene may be inactive. This is further substantiated by the fact that RT-PCR analysis on RNA samples from patients as well as controls could not detect any TUBB4Q expression. In 1988, Little and Seehaus proposed an evolutionary model for tubulin evolution suggesting that TUBB4Q might have become a pseudogene quite recently and is still part of an ongoing evolutionary process towards the generation of a new functional β -tubulin gene. The identification of a tubulin pseudogene in the region should not rule out the possibility of it being causative in FSHD. The reactivation of a pseudogene as a mechanism for human disease is not uncommon and has been reported previously, for example in atypical growth hormone insensitivity (Metherell *et al.*, 2001) and in Coppock-like cataract (Brakenhoff *et al.*, 1994). It was also reported in yeast that there specifically appears to be a mechanism for conditionally "resurrecting" disabled genes as an evolutionary buffer to environmental fluctuation

(Harrison and Gerstein, 2002). In FSHD, it is possible that the expression of TUBB4Q protein might interfere with that of the normally expressed tubulin proteins.

2.2.2.6.3 FSHD region gene 2 (FRG2)

A third gene linked to the D4Z4 locus was revealed to be in even closer proximity to D4Z4 than the two previously described genes. FRG2 was identified exclusively by means of *in silico* gene prediction programs and subsequent expression studies (Van der Maarel, unpublished data). These computer aided exon prediction programs revealed the presence of four putative exons within a 3 kb interval approximately 37 kb proximal to D4F104S1, which is recognized by p13E-11. The studies also revealed two putative polyadenylation signals as well as a strong promoter sequence preceding the FRG2 gene. The gene contains four exons and is transcribed into an mRNA transcript of 2,084 bp, which in turn encodes a 278 amino acid protein. Homology and motif searches revealed the presence of two potential nuclear localization signals (NLS) as well as a peroxisomal targeting signal (PTS1) in the FRG2 sequence. This would suggest that FRG1 encodes a nuclear protein. Closely related sequences to FRG2 have been identified and mapped to chromosomes 1, 4, 8, 10, 18 and 20 respectively.

Whilst investigating the expression patterns of FRG2 in myoblast cultures, it was observed that the protein was only expressed in differentiating myoblasts of FSHD patients and not in control myoblasts (Van der Maarel, unpublished data). The expression was also observed to be absent in most of the other human tissues tested. Fibroblasts of controls and patients undergoing forced myogenesis through infection of an adenovirus carrying the murine *MyoD* gene revealed strong FRG2 expression. This gives an indication that FRG2 at least plays a role in muscle development and hence makes it an attractive candidate for FSHD.

2.2.2.7 Sporadic cases of FSHD

De novo cases are encountered more often than expected and generally have smaller residual fragments with matching increased clinical severity. Studies in the past have reported that up to 30% of FSHD cases investigated harboured new mutations (Padberg *et al.*, 1995; Lunt *et al.*, 1995; Tawil *et al.*, 1996; Zatz *et al.*, 1998). Present findings clearly illustrate a higher frequency than previously reported, i.e. 10% by Lunt and Harper (1991). Griggs *et al.* (1993) and Wijmenga *et al.* (1992) clearly demonstrated smaller sized deletion fragments in sporadic cases.

An interesting observation was that the *de novo* patients predominantly inherited their deletion fragments along with their maternally inherited chromosome 4. In 1994, Wijmenga *et al.* reported that in seven cases of *de novo* patients investigated, five displayed deletions of maternal origin. Although the numbers of patients were small, the results suggested a slight preference for the deletion to take place on one of the maternal chromosomes (Wijmenga *et al.*, 1994).

Interestingly, Lunt reported at the 44th ENMC International Workshop (1998) that in 128 clinically *de novo* cases investigated, as many as 30% were associated with parental somatic mosaicism or possible pre-mutation. This lays the foundation for interesting and unknown mutation mechanisms operating at the mitotic as well as meiotic levels.

2.2.2.8 Anticipation

In terms of FSHD, patients in subsequent generations that succumb to the effects of anticipation generally display symptoms at an earlier onset and/or increased severity in muscle atrophy (Zatz *et al.*, 1995, Lunt *et al.*, 1995, Tawil *et al.*, 1996). The clinical spectrum often encompasses extra-muscular involvement (section 2.2.1.1.3). The notion that the phenomenon of anticipation is observed in generations that have a history of FSHD has been a source for debate and controversy in the past and to date still cannot be excluded from the phenotypic spectrum.

Zatz *et al.* (1995) were the first to report the possible presence of anticipation in FSHD. They investigated 34 FSHD families based on the necessary clinical, neurological and genetic criteria. On average, the onset of clinical signs occurred significantly earlier in the affected offspring as compared with their affected parents. In the same year, Lunt *et al.*, (1995) published data demonstrating that, even though Southern blot analysis confirmed that the size of the deletion fragment remained constant over the successive generations, it seemed clear that the age at onset of FSHD became younger, particularly across three generations. A further study enforcing the idea of anticipation was reported by Tawil *et al.* (1996). This investigation, however, reflected more on the symptomatic levels of the individuals affected by the disorder rather than ages of onset. It was suggested that measurements from these previous studies are subjective, since as many as one-third of patients were asymptomatic at the age of diagnosis and ascertainment bias played a role. In order to determine relative disease severity, quantitative isometric myometry (QMT) and standardised scores with respect to age, gender and height (using a large normative database) were used. This allowed for a more direct and reliable means for determining age of FSHD onset. Based on an investigation of 23 families and QMT scores, Tawil *et al.*

(1996) illustrated that offspring were more severely affected within the statistically significant ranges, and hence came to the conclusion that anticipation was present in the families investigated.

Anticipation is most commonly associated with Kennedy's disease, Myotonic dystrophy and Fragile X syndrome (Warren and Nelson, 1994, Lieberman and Fischbeck, 2000). Based on the current genetic model, the above are characterised by a trinucleotide repeat expansion where the number of repeats increases with each subsequent generation (La Spada *et al.*, 1994). Since FSHD related deletion fragments are too large to be straightforwardly amplified via the polymerase chain reaction (PCR), Southern blot analysis is the only means to detect and analyse the fragments. If clinical anticipation is observed in FSHD, as suggested by the Zatz *et al.* (1995), Lunt *et al.* (1995) and Tawil *et al.* (1996), then a novel molecular model must be inferred. In addition, the mentioned authors studied affected parent-offspring pairs from multiple generations (Flanigan *et al.*, 2001). In order to remove the possibilities for bias, which may occur in studies of multiple families, Flanigan and colleagues are currently investigating several parent-offspring pairs and grandparent-parent-offspring sets within a large single kindred.

2.3 OBJECTIVES OF THIS STUDY

The broad aim of the FSHD research programme is to elucidate the pathogenesis of the disorder in the South African population. This project is aimed at determining the translocation frequency of the D4Z4 repeat arrays on chromosome 4q and those originating from chromosome 10q; and simultaneously verify the chromosomal origin of specific deletion fragments. The data generated in the project will ultimately be used in a comparative study with data generated from the Dutch population.

2.3.1 Specific aims

1. Optimisation of the *Bgl* II - *Bln* I dosage test (Van der Maarel *et al.*, 1999) via non-radioactive protocol.
2. Investigate 80 to 100 randomly selected individuals from South African FSHD families, in order to verify the presence of translocation events.

CHAPTER THREE

MATERIALS AND METHODS

The *Bln* I - *Bgl* II dosage test was utilised to investigate the presence of translocation events in randomly selected individuals from FSHD families. This procedure involves the digestion of gDNA, which is followed by a Southern blot and hybridisation with non-radioactively labelled probe p13E-11. All materials and reagents used were analar grade, from the various supplies, as indicated for each reagent. This study has been approved by the ethics committee of Potchefstroom University for CHE with approval number 02M07 for the study entitled "Molecular analysis of the South African facioscapulohumeral muscular dystrophy (FSHD) population". It was also approved previously by the ethics committee of the University of Pretoria with approval number 91/96. Informed consent was obtained from all individuals, or their legal guardians, prior to enrolment in the study.

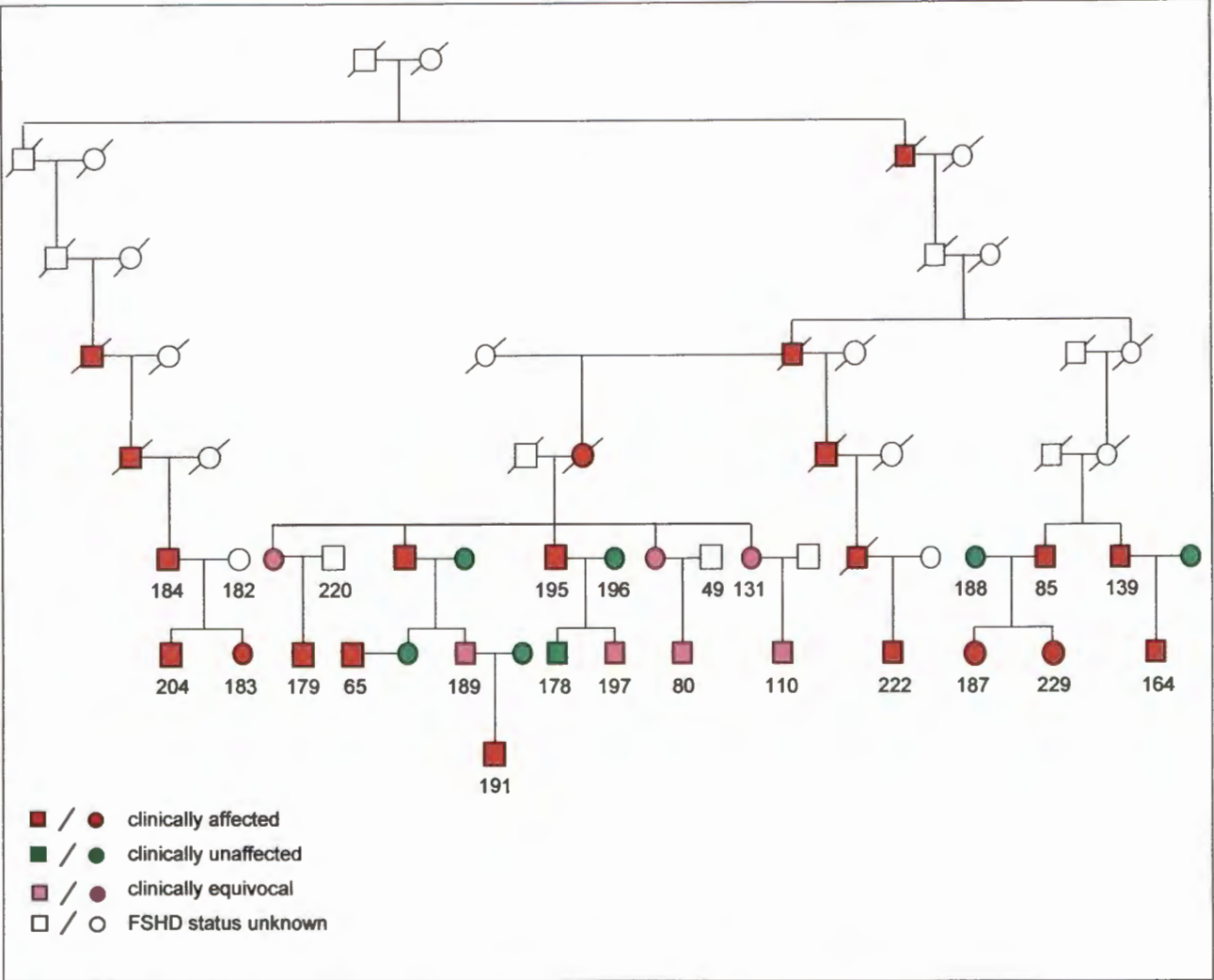
3.1 FSHD FAMILIES INVESTIGATED

As described earlier, the clinical diagnosis of FSHD is challenging due to the subtle manifestation of the phenotype in some cases. For this reason patients enrolled in the programme were only clinically diagnosed by one neurologist, Dr C-M Schutte (University of Pretoria) to ensure that the clinical criteria for the diagnosis were implemented consistently. A total of 90 individuals were included in this study.

3.1.1 Family F10

Family F10 is the largest family included in the South African FSHD programme. This family has been subdivided into several sub-families. Figure 3.1 represents an excerpt of family F10 that includes the DNA numbers of the individuals that were analysed in this study. Individual 222, the proband of family F10, was diagnosed on a clinical level with extreme facial weakness and winging of the scapula. His extremities, however, displayed minimal muscle weakness. A total of 24 individuals from family F10 were included in this study.

Figure 3.1: Excerpt from a pedigree of family F10

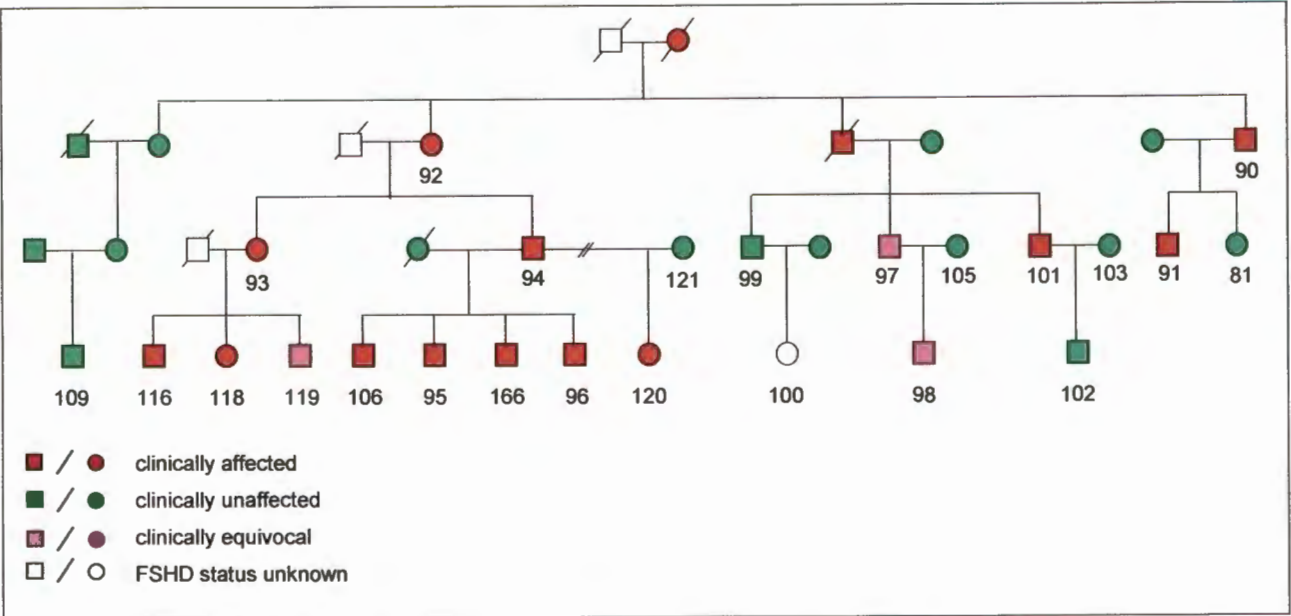


Pedigrees compiled by A. van der Merwe. The numbers indicate the individuals investigated in this study.

3.1.2 Family F20

Family F20 is the second biggest family included in the FSHD programme. Figure 3.2 presents an excerpt of the pedigree of family F20 and the 24 numbered individuals selected for this study. The proband, individual 93, is an elderly female who was clinically diagnosed to be FSHD positive. A total of 24 individuals were selected for analysis in this study.

Figure 3.2: Excerpt from a pedigree of family F20

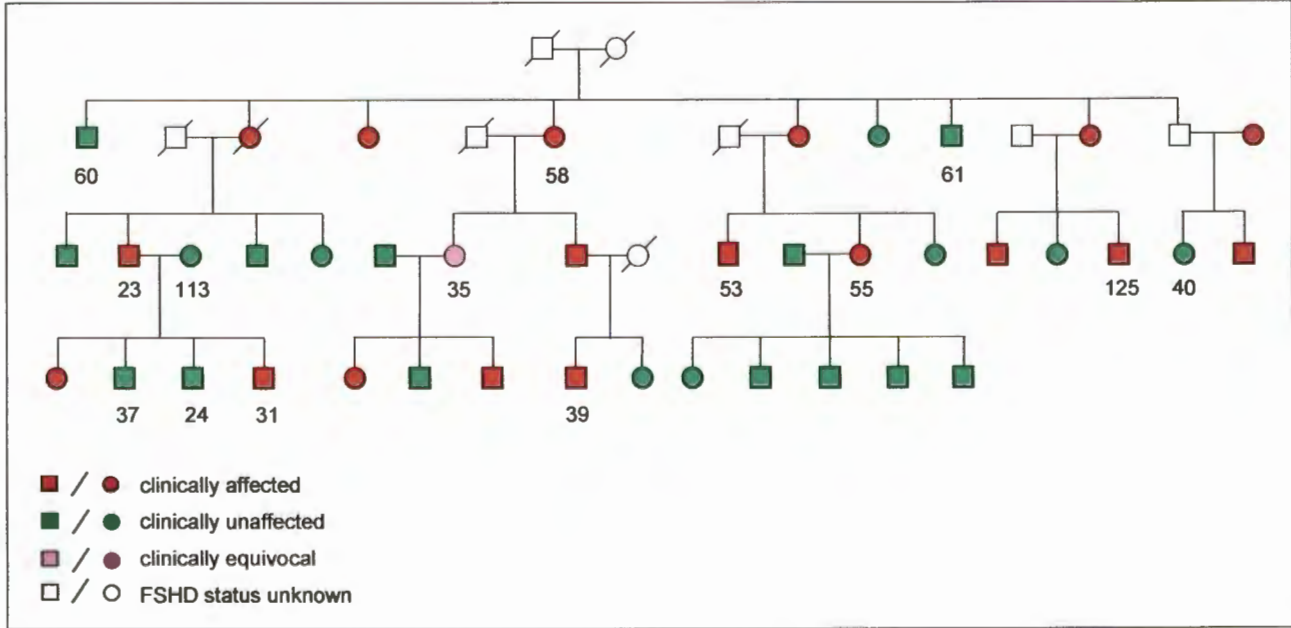


Pedigrees compiled by A. van der Merwe. The numbers indicate the individuals investigated in this study.

3.1.3 Family F30

A 65 year old male was the first member of family F30 to be diagnosed with FSHD. He presented with distinct facial weakness and significant atrophy and weakness of his shoulder girdle muscles. The hip region also displayed weakness. The patient is indicated as individual 23 in the pedigree of this family (Figure 3.3). The excerpt represents four generations of family F30 including the 14 members investigated in this study.

Figure 3.3: Excerpt from a pedigree of family F30

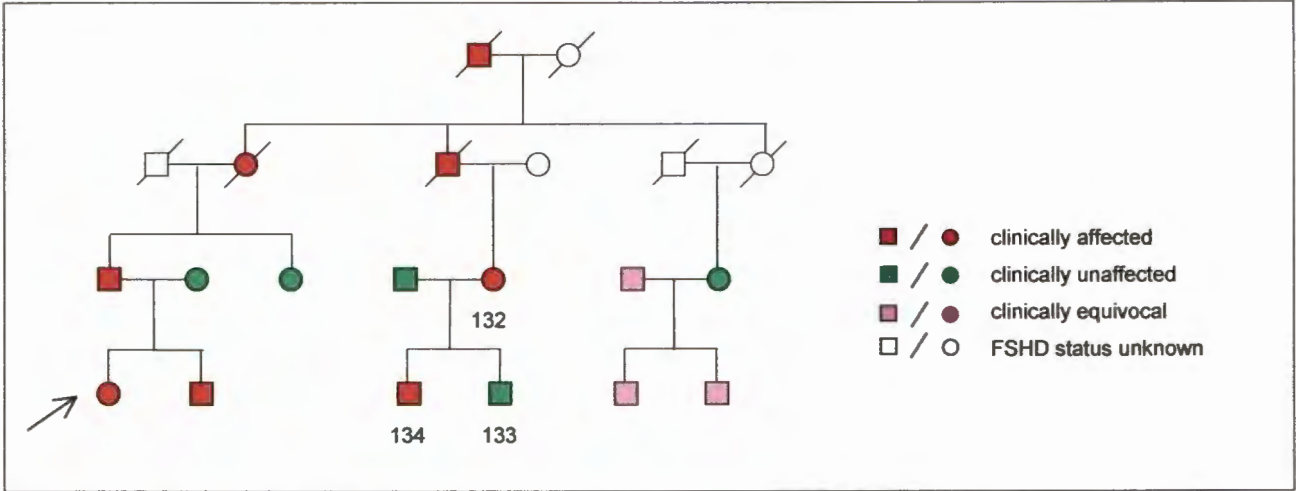


Pedigrees compiled by A. van der Merwe. The numbers indicate the individuals investigated in this study.

3.1.4 Family F40

Family F40, displayed in Figure 3.4, includes the proband, a middle aged female with extreme winging of the scapula due to weakness of the muscles around the scapula. This individual has a distinct waddling gait due to the weakness of pelvic girdle muscles. The muscles of her face and upper arms were, however, only mildly affected. A total of three individuals were analysed in this study.

Figure 3.4: Excerpt from a pedigree of family F40

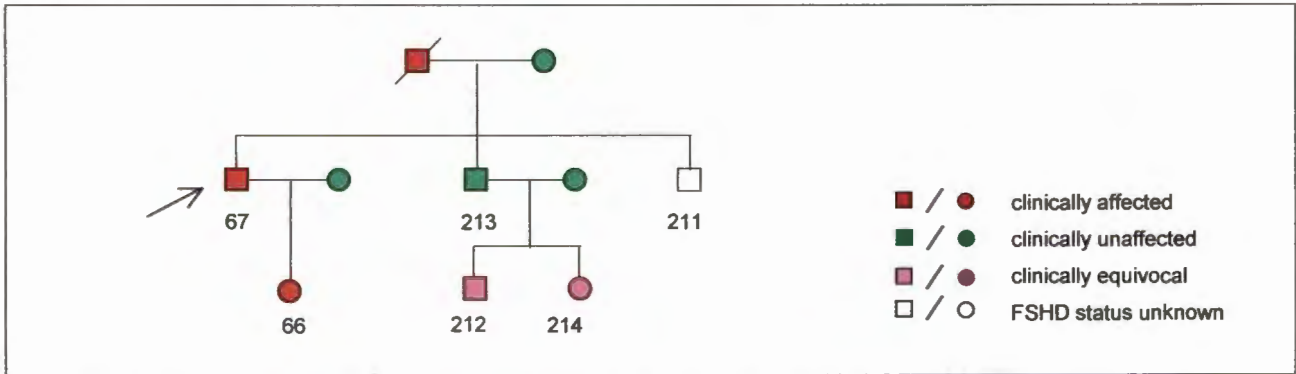


Pedigrees compiled by A. van der Merwe. The numbers indicate the individuals investigated in this study.

3.1.5 Family F60

The proband of family F60 (67) is a middle aged male with atrophic pectoralis major muscles and facial muscles. This family consists of 75 individuals of which six were included in this study.

Figure 3.5: Excerpt from a pedigree of family F60



Pedigrees compiled by A. van der Merwe. The numbers indicate the individuals investigated in this study.

3.1.6 Random individuals

Apart from the individuals included in the above families, additional individuals were selected for this study. These individuals do not belong to a specific FSHD family, but were individually referred for analysis. This group consists of the following individuals: 69, 70, 71, 73, 75, 76, 77, 205, 206, 207, 221, 236, 402, 458, 459, 480, 481.

3.2 THE *Bgl* II – *Bln* I DOSAGE TEST

Prof. Silvère van der Maarel, Department of Human Genetics, Leiden University, Medical Center kindly donated probe p13E-11 and supplied an optimised Southern blot protocol for the South African research programme. gDNA was previously isolated and quantified for the individuals included in this study.

3.2.1 Restriction enzyme digestion of gDNA

Isolated gDNA in the order of 2.5 to 5.0 micrograms (μg) was digested in a double digestion reaction with 20 U of each of the restriction enzymes *Bgl* II and *Bln* I [Roche and Amersham]. The restriction enzyme digestion was carried out overnight at 37°C in the presence of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 100 mM NaCl, 1 mM dithiothreitol [Roche] or 20 mM Tris-HCl (pH 8.5), 10 mM MgCl_2 , 100 mM KCl, 1 mM dithiothreitol [Amersham] and made up to a final volume of 30 microlitres (μl).

3.2.2 Agarose gel electrophoresis

The product of the double digestion was first analysed via agarose gel electrophoresis on a mini submarine agarose gel. A 1% agarose gel was cast utilising 0.4 g molecular grade agarose [Promega] and made up to 40 ml with 1X TBE buffer (89.15 mM Tris base [USB] (pH of 8.0-8.3), 88.95 mM boric acid [USB]; 2.498 mM Na_2EDTA [ACE]). The agarose gel was supplemented with 1.268 μM EtBr [Sigma[®]¹] to allow for fluorescent detection of the DNA. Two and a half microlitres of the restriction endonuclease digestion reaction was mixed with 1 μl of a 2X loading buffer (0.04% Orange G [Sigma[®]] and 50% glycerol) and loaded into the wells of the gel. The mini agarose gel was electrophoresed in 1X TBE buffer at 100 V (10 V.cm^{-1}) for 30 minutes and digested DNA visualised via UV transillumination in order to ascertain digestion of the gDNA.

¹ Sigma[®] is a registered trademark of sigma Chemical Company, St louis, MO, U.S.A.

Upon verification of complete digestion, the residual restriction endonuclease product was subsequently loaded onto a 0.8% maxi agarose gel containing 1.268 μM EtBr [Sigma®]. Gibco 1 kb molecular weight marker (MWM) was also loaded alongside the digestion products to enable fragment size determination following detection. The maxi agarose gel was electrophoresed in 1X TBE buffer at 1.5 V.cm^{-1} for a minimum of 22 hours.

3.2.3 Southern blot analysis

Southern blot analysis, utilising probe p13E-11 was used to detect the fragments generated via the dosage test. This protocol was based on the original method described by Southern (1975).

3.2.3.1 gDNA transfer

The electrophoresed gel was carefully removed from the submarine gel apparatus and analysed on a UV transilluminator. After visualisation of the digested gDNA, the DNA was nicked via depurination in 0.25 N HCl for 10 minutes and denatured in freshly made up alkaline blotting solution (0.4 M NaOH, 1.6 M NaCl) during two 20 minute incubations. The DNA was then transferred overnight to Hybond™¹ N⁺ membrane [Amersham] using capillary action and alkaline blotting solution. After ca. 14 hours, the membrane was neutralised for 5 minutes in 2X SSC (0.15 M NaCl, 15 mM Na-citrate), and 0.2 M Tris-HCl (pH 7.0) and the DNA fixed to the membrane by baking for 2 hours at 80°C. The membrane was then ready for hybridisation and DNA detection procedures.

3.2.3.2 Amplification of p13E-11

Probe p13E-11 was cloned into the pBluescript plasmid and thus M13 forward and reverse primers were used for the amplification process. The PCR reactions were prepared by adding 1 X PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl_2), 200 μM each of the dNTPs [Promega], 10 pmol of the M13 forward and reverse primers and 0.5 units of Taq DNA polymerase [Promega]. Four hundred and fifty nanograms (ng) of plasmid were added and the reactions made up to a total volume of 25 μl . Finally a drop of mineral oil was added to overlay the reactions and prevent evaporation.

¹ Hybond™ is a trademark of Amersham International plc., Buckinghamshire, England.

The PCR programme consisted of denaturation at 94°C for 10 minutes, followed by 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. In order to ensure full-length products, a final extension of 7 min at 72°C was included.

3.2.3.3 Purification of probe p13E-11

The amplified probe was loaded onto a 1% low-melt agarose (FMC) gel whereafter the Wizard^{®1} SV Gel and PCR Clean-Up System was utilised to purify probe p13E-11. The fragment was excised from the gel and dissolved in the presence of Membrane Binding Solution, consisting of guinidine isothiocyanate. The mixture was vortexed and incubated at 65°C for 10 minutes. A SV mini-column was placed in a collection tube and the dissolved gel mixture was transferred to the column and incubated for 1 minute at room temperature. The column was centrifuged at 10,000X g for 1 minute and the liquid in the collection tube discarded. A total of 700 µl of Membrane Wash Solution, diluted with 95% ethanol, was added to the column before centrifugation at 10,000X g for 1 minute. The collection tube was emptied and a second batch of Membrane Wash Solution added to the column followed by centrifugation at 10,000X g for 5 minutes. The column was transferred to a clean 1.5 ml Eppendorf^{®2} tube and 50 µl of ddH₂O was added to the centre of the column. The DNA was then eluted via centrifugation at 10,000X g for 1 minute. The purified products were subsequently quantified and concentrations determined via spectrophotometric analysis on the BioPhotometer (Eppendorf[®]).

3.2.3.4 Labelling and quantification of probe p13E-11

Two different labelling and quantification strategies were undertaken for the labelling of the purified probe and will be discussed in sections 3.2.3.4.1 to 3.2.3.6.

3.2.3.4.1 Labelling utilising digoxigenin-dUTP

Labelling of probe p13E-11 was performed utilising both the DIG High Prime DNA Labelling and Detection Starter Kit II [Roche] and the PCR DIG Probe Synthesis Kit [Roche].

¹ Wizard is a registered trademark of Promega Corporation, Madison, WI, U.S.A.

² Eppendorf is a registered trademark of Eppendorf, Hamburg, Germany.

3.2.3.4.1.1 Random primed labelling of probe

The DIG High Prime labelling kit utilises digoxigenin-dUTP and probes are generated according to a random primed labelling technique. A total of 1.2 µg of probe DNA was added to ddH₂O to a final volume of 16 µl. The probe DNA was denatured for 10 min at 97°C and mixed with 4 µl of DIG-High Prime Solution. The reaction was incubated overnight at 37°C and subsequently stopped by the addition of 2 µl of 0.2 M EDTA (pH 8.0). The labelled probe was quantified as described in section 3.2.3.5.

3.2.3.4.1.2 PCR labelling of probe

The PCR DIG Probe Synthesis Kit utilises PCR for the direct labelling of DNA via the incorporation of DIG-dUTP into the PCR product. This kit is especially designed to generate highly sensitive hybridisation probes suitable for the detection of low (single) copy target sequences. The PCR reactions were prepared in a total of 50 µl. A total of 10 pg of probe DNA was mixed with 5 µl of PCR buffer containing MgCl₂, 5 µl PCR DIG labelling mixture, 5 pmol of each of the M13 forward and reverse primers and 2.6 U Expand™¹ High Fidelity Enzyme mix. The reagents were mixed and centrifuged to collect the sample at the bottom of the tube.

The PCR programme consisted of: denaturation at 94°C for 10 minutes, and 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. A final extension of 7 min at 72°C ensured full length products.

3.2.3.4.2 Labelling utilising biotin-N4-dCTP

Labelling was performed utilising the Detector™² Random Primer DNA Biotinylation Kit (Kirkegaard and Perry Laboratories [KPL]). One hundred ng of template DNA (already isolated probe or MWM) was diluted to a final volume of 24 µl with ultra high quality water, which was mixed with 15 µg 2.5X random primer mix. The DNA was denatured at 95°C for five minutes and placed immediately onto ice. The mixture was subsequently made up to a final volume of 50 µl by the addition of 5 µl of 10X dNTPs and 10 U of Klenow polymerase, followed by a 4 hour incubation at 37°C to allow labelling of the probe. The dNTP mixture consists of standard dNTPs as well as an equal concentration of

¹ Expand is a trademark of a Member of the Roche Group, Mannheim, Germany.

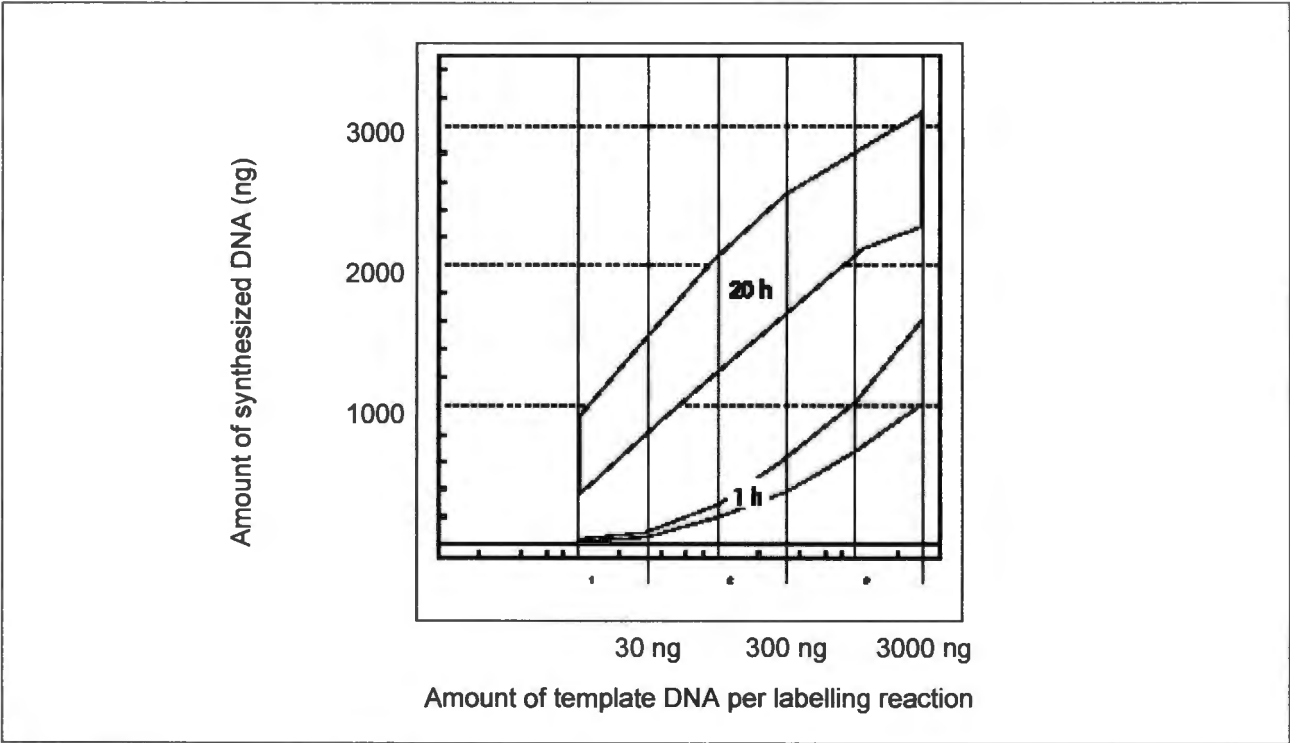
² Detector™ is a trademark of Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD, U.S.A.

biotin-N₄-dCTPs. An addition of 5 µl of stop buffer terminated the labelling reaction. The labelled probe was quantified as described in section 3.2.3.6.

3.2.3.5 Quantification of probe yield labelled with digoxigenin-dUTP

The probe yield after labelling was determined to ensure reproducible hybridisation results. A 1 ng.µl⁻¹ dilution was prepared from the labelled probe, according to the expected yield of synthesised DNA as depicted in Figure 3.6.

Figure 3.6: Yield of DIG-labelled DNA



Adapted from DIG High Prime Labelling and Detection Starter Kit II, 2002.

A dilution series was prepared as presented in Table 3.1. After the preparation of the dilution series, 1 µl of each dilution (tubes 2-9) was applied to a nylon membrane and fixed by baking for 30 min at 80°C. The DIG Wash and Block Buffer Set [Roche] was utilised for the immunological detection of all the hybridisations. The membrane was incubated in Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) for 2 min at room temperature and subsequently in 10 ml Blocking solution for 30 min. Subsequent to blocking, the membrane was incubated in 10 ml Antibody solution for 30 min and washed twice with 10 ml Washing buffer (0.1 M Maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween^{®1} 20, pH 7.5) for 15 min, after which it was equilibrated in 10 ml Detection buffer (0.1 M Tris-HCl [pH 9.5),

¹ Tween is a registered trademark of ICI Americas, Inc., U.S.A.

0.1 M NaCl) for 5 min. The membrane was placed in a hybridisation bag and incubated with 0.1 ml CSPD^{®1} for 5 min. The excess liquid was removed and the edges of the hybridisation bag sealed. Subsequently, the membrane was exposed to X-ray film for 15 min.

Table 3.1: Preparation of dilution series

Tube	DNA (μl)	From tube #	DNA Dilution Buffer	Dilution	Final concentration
1	-	Diluted original	-	-	1 ng.μl ⁻¹
2	5	1	495	1:100	10 pg.μl ⁻¹
3	15	2	35	1:3.3	3 pg.μl ⁻¹
4	5	2	45	1:10	1 pg.μl ⁻¹
5	5	3	45	1:10	0.3 pg.μl ⁻¹
6	5	4	45	1:10	0.1 pg.μl ⁻¹
7	5	5	45	1:10	0.03 pg.μl ⁻¹
8	5	6	45	1:10	0.01 pg.μl ⁻¹
9	0	-	50	-	0 pg.μl ⁻¹

Adapted from DIG High Prime DNA Labeling and Detection Starter Kit II Instruction Manual, 2002.

3.2.3.6 Quantification of probe yield labelled with biotin-N₄-dCTP

Via the quantification step the precise concentration of the probe was determined in order to calculate the required amount for the hybridisation reaction. A series of two-fold dilutions of a 1/200 diluted labelled probe and undiluted biotinylated DNA standard was constructed prior to dot blot quantification. The dot blot was prepared by spotting 1 μl of each of the dilutions onto a dry membrane, previously pre-treated in 5X SSC for 10 minutes. After allowing the dots to dry for 10 minutes, the DNA was cross-linked to the membrane by baking for 30 minutes at 80°C.

The detection procedure was performed using AP-streptavidin (AP-SA) and CDP-Star^{®2}. Prior to detection with the DNADetector™ Genomic Southern Blotting Kit [KPL], the blocking/diluent solution was prepared by diluting 5X Detector Block Solution 1:5 in distilled water and adding 0.2% Detector Block Powder. The solution was heated to aid in dissolving of the powder. The membrane was subsequently incubated for 30 minutes in the blocking/diluent solution at room temperature, followed by incubation for 30 minutes in a fresh solution of AP-SA diluted 1:10,000 in blocking/diluent solution. The membrane was washed three times for 5 minutes in a 1X Phosphatase Wash Solution, followed by

¹ CSPD is a registered trademark of Tropix, Inc., Bedford, MA, U.S.A.

² CDP-Star[®] is a registered trademark of Tropix Inc., Bedford, MA, U.S.A.

rinsing twice for 2 minutes in 1X Phosphatase Assay Buffer. Subsequently, the membrane was drained and incubated for 5 minutes in CDP-Star® Chemiluminescent Substrate. After placing the membrane in a hybridisation bag and exposing it to X-ray film, the relative specific activity of the labelled probe was determined by calculating the ratio of probe to standard (P/S).

3.2.3.7 Hybridisation with the digoxigenin-dUTP labelled probe

The appropriate hybridisation temperature was calculated according to the GC content, the length of probe p13E-11 and the degree of homology of the probe to the template (more than 80%). The hybridisation temperature (T_{opt}) was calculated according to Equations 3.1 and 3.2, as suggested by the manufacturer.

Equation 3.1: Annealing temperature of probe to target

$$T_m = 49.82 + 0.41(\%G+C) - (600/l)$$

Equation 3.2: Optimised annealing temperature of probe to target

$$T_{opt} = T_m - 20 \text{ to } 25^{\circ}\text{C}$$

As depicted in Table 3.2, probe p13E-11 is 788 bp and has a 39% GC content. The hybridisation temperature for probe p13E-11 was therefore calculated to be 40 - 45°C.

Table 3.2: gDNA target sequence of probe p13E-11

1	ggggatctat	acagcactca	tcaaatctag	catttaaaga	ctggctcagt	aaagggggat
61	accgacagca	atagtccagg	ctatagattc	taggtgcttc	acaaacacat	tcccaaattg
121	gacttcgctg	tgtttccaag	ttaaagagaa	ttttttctca	atgtatttat	attttctctg
181	tatatTTTct	tccccagttg	gctgtctgtg	gtattgcagt	ttcactagta	gattctataa
241	cactcatctt	tcttctcagc	agacacaaac	tgtcatctat	atgactcctg	ctgtagcatc
301	agcactccac	atcaggagag	aaagaatcta	gtcattagac	aatttatcat	catgtcctac
361	atttcaacac	atattctact	gttttaattc	tctcctgaag	gagatactaa	aaagccaaca
421	ttttctcatt	agcccagtta	ctgttctggg	tgaaaaaata	aactgcaggg	gagttgggggt
481	aagccagact	tcatcaaatt	tctgcaccaa	tgaaaaaaaa	atttacaatg	gacaggctaa
541	aaaccttatt	aaacgtcacg	gacaaggcca	gagtttgaat	atactgtgga	gaaaaacact
601	ccagtgcaaa	ctgtttccag	aaagcctact	tctatTTTcc	ttgctgtagt	catctctgat
661	ttcctgtctt	atgtttattc	tactctgcaa	tcccctaagg	ctttttctac	agaggaacga
721	atcttaaagt	gcattcgaac	tcacaggcaa	aatcctccca	gaatcttgct	ccctcccatg
781	agaacata					

DNA sequence was retrieved from GenBank with accession number AF117653 as reported by Gabriels *et al.* (1999).

The hybridisation process was performed in hybridisation bottles in a Techne hybridisation oven. A total of 36 ml of DIG Easy Hyb solution (supplied in the DIG High Prime DNA Labelling and Detection Starter Kit II [Roche]), was preheated to the hybridisation temperature. The membrane was pre-hybridised for 30 min. Twenty five $\text{ng} \cdot \mu\text{l}^{-1}$ DIG-labelled DNA probe was denatured for 5 min at 97°C , and subsequently added to the pre-heated DIG Easy Hyb solution. The hybridisation reaction was performed at the calculated hybridisation temperature for 16 to 18 hours.

3.2.3.8 Hybridisation with the biotin- N_4 -dCTP labelled probe

The membrane, to which the DNA was fixed, was prehybridised to prevent background binding of the probe. The membrane was incubated for one hour at 42°C in a pre-hybridisation solution prepared from the Formamide Hybridisation Buffer included in the kit [KPL] and $200 \mu\text{g} \cdot \text{ml}^{-1}$ sheared, denatured salmon sperm DNA. The biotinylated probe was denatured for 10 minutes at 95°C and added directly into the pre-hybridisation solution at the recommended final concentration of $50 \text{ ng} \cdot \text{ml}^{-1}$. Incubation with agitation was performed at 42°C for 16 to 18 hours.

3.2.3.9 Stringency washes for digoxigenin-dUTP labelled probe

After hybridisation the membrane was washed to remove excess probe. The membrane was washed twice for 5 min in low stringency wash buffer, consisting of 2 X SSC and 0.1% SDS, at room temperature. A second wash with high stringency wash buffer, consisting of 0.5 X SSC and 0.1% SDS, prewarmed to 65°C , was performed twice for 15 min.

3.2.3.10 Stringency washes for biotin- N_4 -dCTP labelled probe

The membrane was removed and washed twice for 15 minutes at room temperature in 2X SSPE [0.3 M NaCl, 20 mM NaH_2PO_4 (pH 7.4), 2 mM EDTA] containing 0.1% SDS. A second wash solution of 0.2X SSPE [0.03 M NaCl, 2 mM NaH_2PO_4 (pH 7.4), 0.2 mM EDTA] containing 0.1% SDS was equilibrated to 55°C and utilised in two 15 minute washes. A final wash was performed for 5 minutes with 2X SSPE at room temperature in order to remove residual SDS.

3.2.3.11 Immunological detection of hybridisation with digoxigenin-dUTP labelled probe

After hybridisation and stringency washes, the membrane was rinsed briefly in Washing buffer and subsequently incubated in 100 ml Blocking solution for 30 min. A total of 20 ml Antibody solution was added and the membrane incubated for 30 min. The membrane was washed twice in 100 ml Washing buffer for 15 min and equilibrated in 20 ml Detection buffer for 5 min. Finally the membrane was incubated in CSPD for 5 min at room temperature and exposed to an X-ray film (Hyperfilm™¹ ECL™²) for 15 to 25 min.

3.2.3.12 Immunological detection of hybridisation with biotin-N₄-dCTP labelled probe

The previously mentioned DNADetector™ Genomic Southern Blotting Kit (KPL) was also utilised for the detection procedure. The first step in the detection process was to incubate the hybridised membrane for 45 minutes in 1X blocking/diluent solution prepared from 5X Detector Block Solution and 1% Detector Block Powder. It was imperative to ensure that all the reagents were dissolved completely. AP-SA conjugate was subsequently diluted in an additional amount of 1X blocking/diluent (1:10,000) and poured over the membrane for a 30 minute incubation, subsequent to removal of the previous 1X blocking/diluent. The membrane was subsequently washed three times for 5 minutes in ready made 1X Phosphatase Wash Solution and rinsed afterwards for 5 minutes in 1X Phosphatase Assay Buffer. Finally the membrane was incubated in CDP-Star® for a period of 5 minutes followed by removal of the CDP-Star® by blotting onto filter paper. Fragments were assayed after exposure of the membrane to X-ray film (Hyperfilm™ ECL™) for approximately 10 minutes.

3.2.3.13 Quantification of detected fragments

The fragments detected by p13E-11 on each of the autoradiographs of the blots were quantified utilising the Adobe Photoshop®³ 6.0 programme. Fragment intensity measurements were recorded at various points across fragments and an average calculated. Background readings were also determined for each of the fragments and taken into account when calculating true intensities of the fragments. A ratio of 4-type to 10-type fragments was subsequently calculated for each individual (see section 4.1.6).

¹ Hyperfilm™ is a trademark of Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, England.

² ECL™ is a trademark of Amersham Biosciences, UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, England.

³ Adobe logo and Photoshop are trademarks of Adobe Systems Incorporated, Seattle, WA, U.S.A.

CHAPTER FOUR

RESULTS AND DISCUSSION

The dosage test was designed to identify the occurrence of translocation events between the repeat sequences originating from chromosomes 4 and 10. Dosage test analysis of the families investigated in this study was performed as described in the previous chapter.

4.1 THE *Bgl* II – *Bln* I DOSAGE TEST

The dosage test is an adaptation of the original Southern blot analysis protocol (Southern, 1975). It encompasses several techniques that include RFLP analysis, electrophoresis, DNA transfer, hybridisation and detection.

4.1.1 gDNA transfer

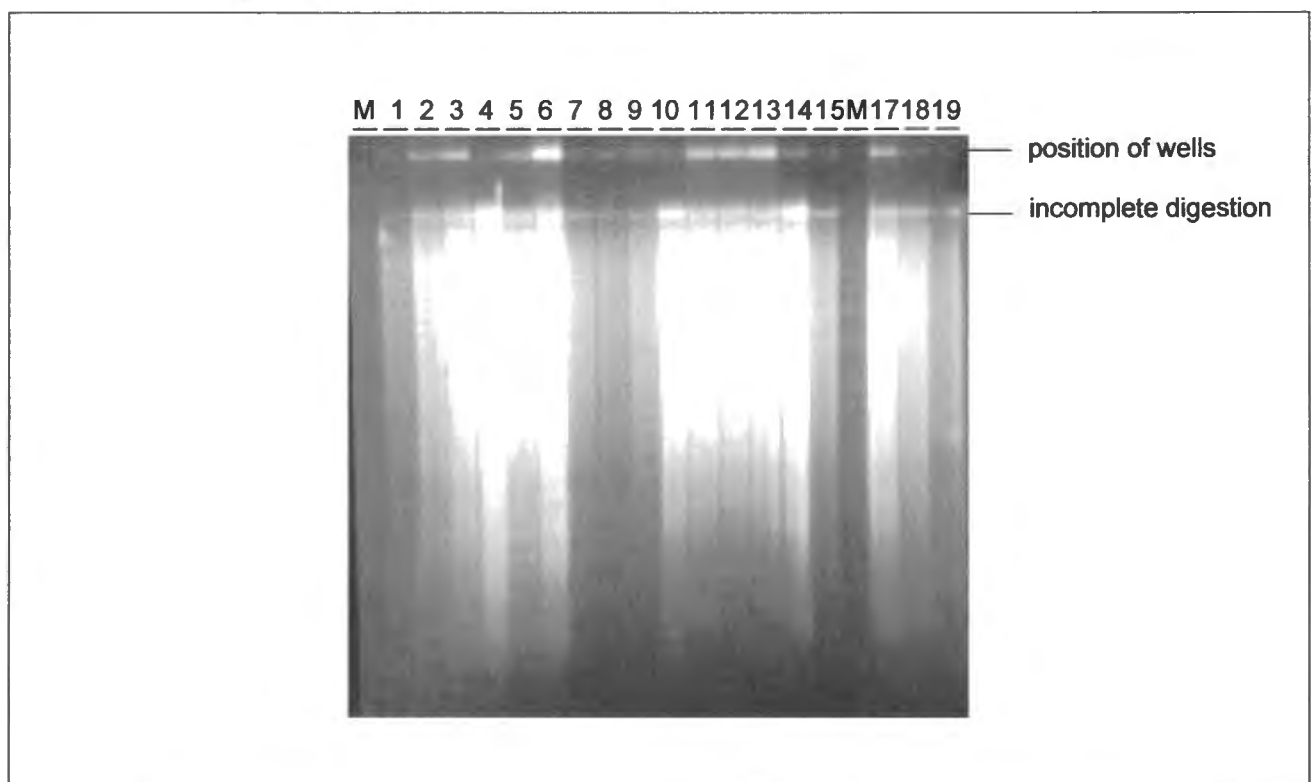
The previously isolated gDNA¹ was digested with restriction endonucleases *Bgl* II and *Bln* I. The digestion reactions were initially performed with 2.5 µg gDNA. Confirmation of complete digestion was obtained via agarose gel electrophoresis and UV transillumination, as indicated in Figure 4.1. A DNA "smear" on the gel indicates that the genome was digested by the restriction enzymes, resulting in a range of DNA fragments of varied sizes. Figure 4.1 illustrates that gDNA was sufficiently digested to enable DNA transfer and detection of 4-type and 10-type fragments. However, the fragments indicated high up on the gel does show that the genome was not fully digested. This is a general problem encountered when digesting high molecular weight DNA (Sambrook and Russell, 2001). It is caused by variations in the local concentration of DNA. The more densely concentrated the DNA, the more inaccessible the internal DNA is for the restriction enzymes. This results in the incomplete digestion, as observed and indicated in Figure 4.1.

An interesting observation from Figure 4.1 is that even though 2.5 µg of individual gDNA was digested, a seemingly broad range of DNA concentrations are visualised amongst the group of individuals investigated. This may have been due to insufficient resuspension or

¹ DNA isolated by A. Van der Merwe.

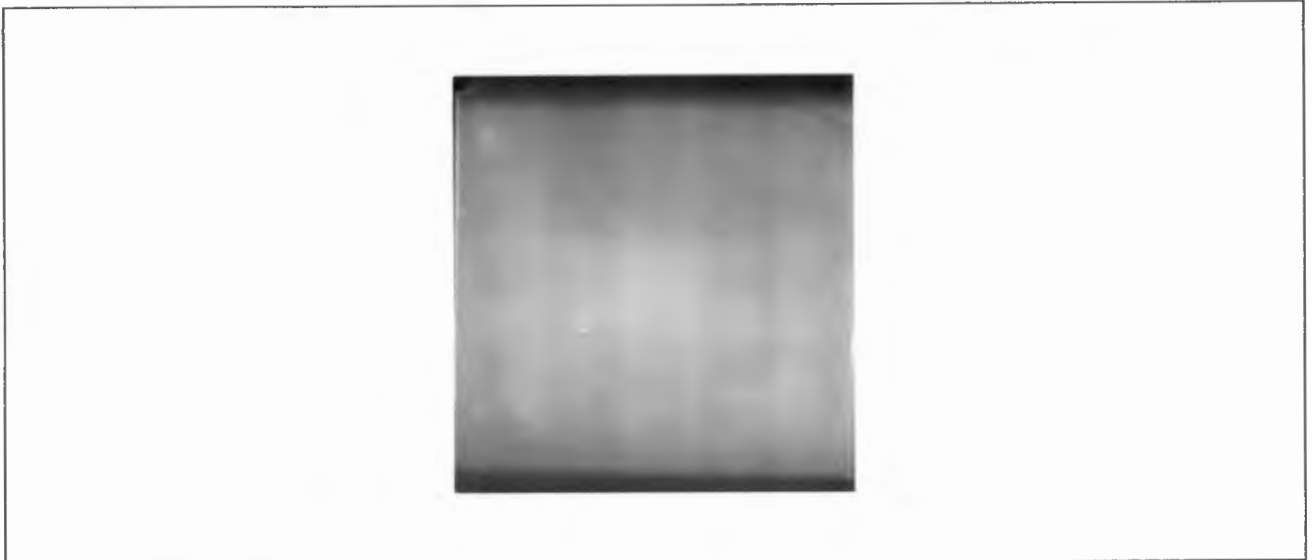
inaccurate aliquotting of the gDNA prior to setting up of the restriction endonuclease reactions. Resuspending of DNA should be performed gently to ensure that DNA is not sheared in the process. Since some of the DNA stock samples tended to be more viscous than others, it may also lead to unequal amounts of DNA aliquots being sampled for the digestion reactions. Whether the discrepancy between the concentrations of DNA observed on the gel and differences in DNA aliquotting are in parallel, is a source for debate. It is clear that since the loaded 1 kb MWM concentration was several fold less than that of gDNA loaded, i.e. 50 ng versus 2.5 μ g of gDNA, the intercalation of EtBr does not allow for its visualisation via UV transillumination.

Figure 4.1: Photographic representation of restriction endonuclease digestion of gDNA



0.8% Agarose electrophoresed at 35 V overnight in 1X TBE. M = 1 kb molecular weight marker, 1-19 represent the lane number of the gel.

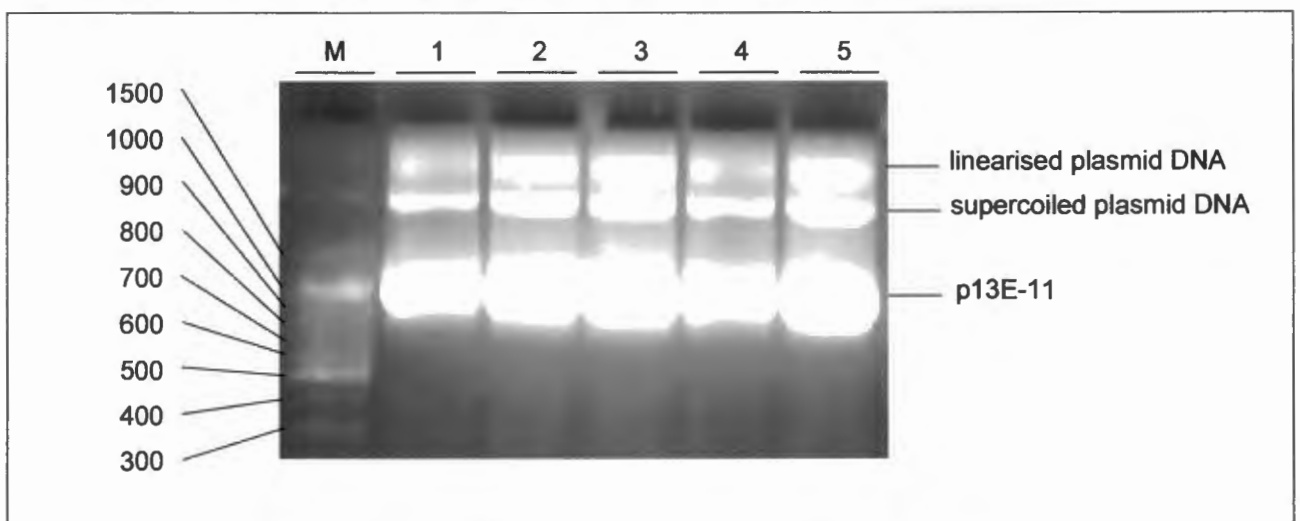
The digested DNA was transferred to the positively charged membrane via alkaline transfer and baked to fix the DNA. The gel was subsequently stained with EtBr and analysed via UV transillumination to verify that the DNA was indeed transferred from the gel to the membrane. Figure 4.2 is an electrophoretic representation of an agarose gel after transfer of the gDNA to the solid support. No DNA fragments can be observed, indicating complete transfer of the gDNA fragments during the blot procedure.

Figure 4.2: Photographic representation of gel confirming DNA transfer

Post transfer gel stained for 30 minutes in 500 ml with ddH₂O containing 2 µg of EtBr.

4.1.2 Preparation of the p13E-11 probe

Probe p13E-11 was prepared for hybridisation via PCR amplification, purification and labelling, utilising both DIG and biotin based procedures. Amplification of p13E-11 was successfully achieved from the PCR protocol described in section 3.2.3.2. In Figure 4.3, a photographic representation of the p13E-11 amplicon is shown, indicating successful amplification.

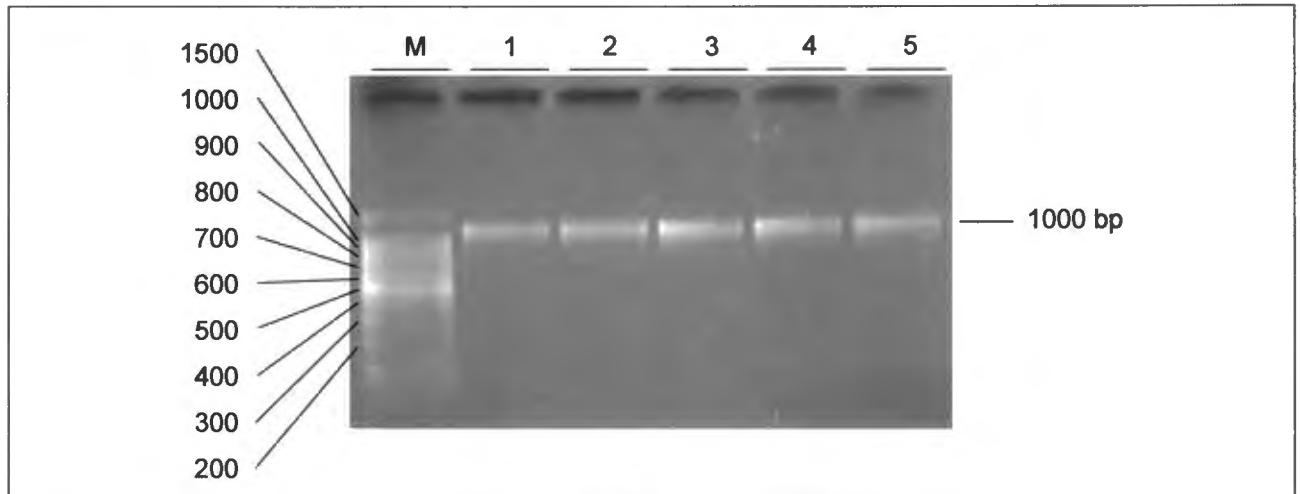
Figure 4.3: Photographic representation of p13E-11 amplicon

1% Agarose gel electrophoresed at 100 V in 1X TBE for 30 minutes; M = 100 bp molecular weight marker.

Subsequent to PCR amplification of p13E-11, the p13E-11 amplicons were excised from the gel and purified via the Wizard® SV Gel and PCR Clean-Up System. The samples

were electrophoresed on a 1% agarose gel, presented in Figure 4.4, to confirm the presence of the purified product. Figure 4.4 clearly illustrates that the process yielded sufficient purified product of the correct size.

Figure 4.4: Photographic representation of the purified p13E-11 amplicon



1% Agarose gel electrophoresed at 100 V in 1X TBE for 30 minutes; M = 100 bp molecular weight marker.

Various samples of the purified probe were pooled. The concentration was determined via a spectrophotometer (BioPhotometer by Eppendorf^{®1}), to be 75 ng.μl⁻¹.

4.1.3 Labelling of probe p13E-11

Probe p13E-11 was labelled via a random primed biotin based method, as well as two other DIG based strategies, the one also being random prime based and the other via direct PCR labelling. The labelling of all procedures, be it biotin or DIG based, appeared to be highly efficient.

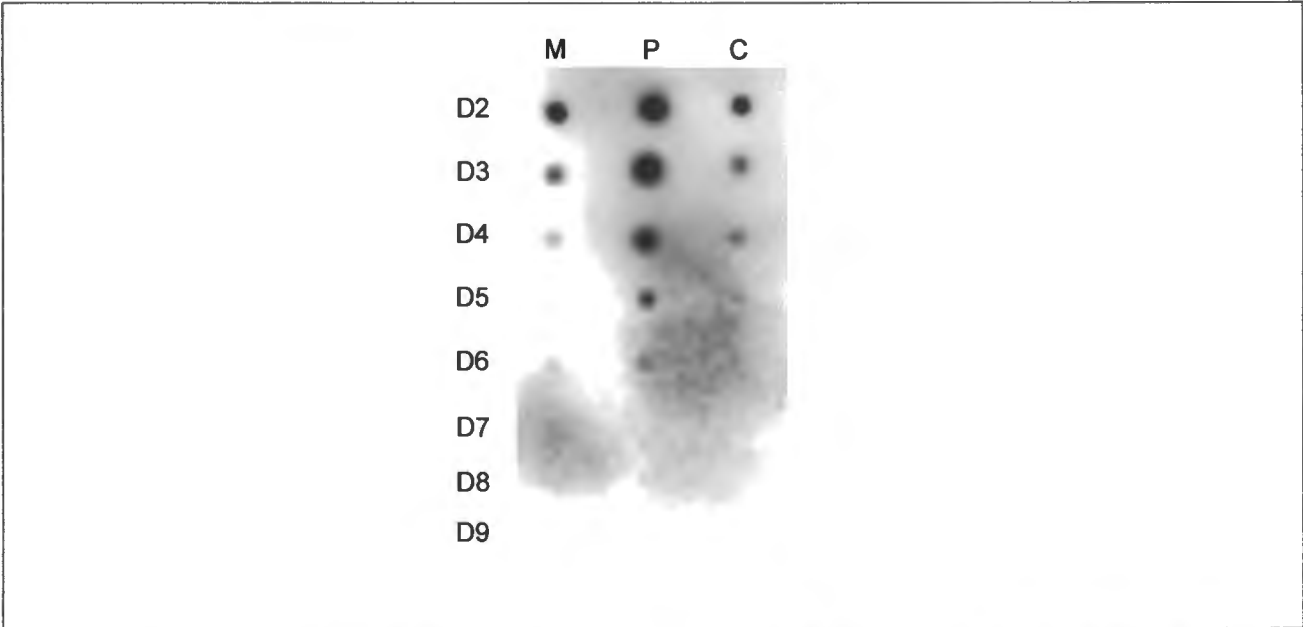
4.1.3.1 DIG labelling of probe p13E-11

Two DIG labelling strategies were performed to optimise DIG incorporation, in order to ensure efficient detection. The random primed method utilising DIG labelled dUTP as well as the PCR based direct labelling methods were successful. Figure 4.5 illustrates the labelling efficiency of p13E-11 and 1 kb MWM via the DIG random primed method. Before performing series dilutions for the dot blot quantification, the DIG random primed method required an estimated concentration of labelled probe as a starting point (see section

¹ Eppendorf is a registered trademark of Eppendorf, Hamburg, Germany.

3.2.3.5). If an accurate estimation was made, then the series dilutions should yield the expected labelled DNA concentrations, and consequently dots up to at least row D6 (see Figure 4.5) should be visible. It is evident from this figure that the probe was labelled efficiently and that the expected amount of labelled DNA was correctly estimated. Since 1,200 ng of purified p13E-11 was used in the labelling reaction, an estimated 1,500 ng of labelled probe is expected.

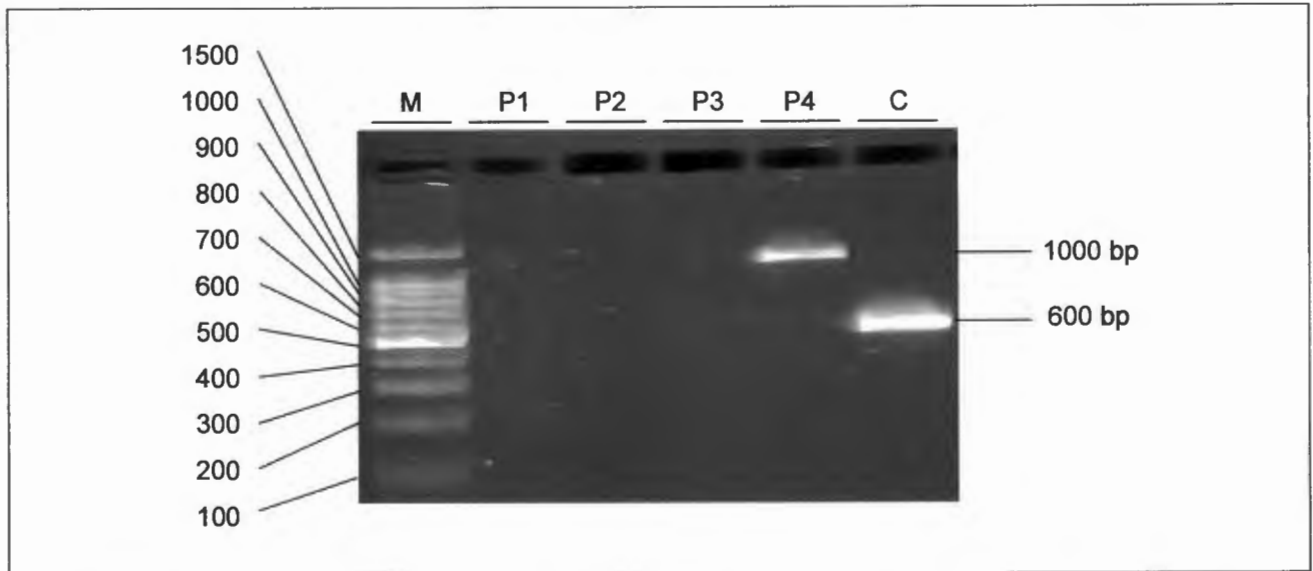
Figure 4.5: Dot blot illustrating labelling efficiency of the DIG random primed method



C = DIG-labelled control DNA, M = DIG labelled molecular weight marker; P = DIG-labelled p13E-11, D1 to D9 = series dilutions set up as described in section 3.2.3.5.

The direct labelling procedure via PCR was also successfully achieved after a brief period of optimisation. As discussed in section 3.2.3.4.1.2, 10 pg.µl⁻¹ of the pBluescript plasmid was suggested to be optimal for amplification and direct dUTP incorporation. However, this initially did not seem to be optimal in the labelling of p13E-11. Amplification was not obtained when using 10 pg.µl⁻¹ of the plasmid, nor was there for a series of even higher plasmid concentrations, as shown in Figure 4.6. However, when altering the ratio of DIG-labelled dUTP:dTTP from the standard 1:3 ratio to that of 1:6, the direct procedure yielded sufficient DIG-labelled p13E-11 as indicated in lane P4 of Figure 4.6. PCR amplification of the control template DNA was also successful and confirmed correct working procedures.

Figure 4.6: Photographic representation of optimised PCR conditions for efficient labelling of p13E-11

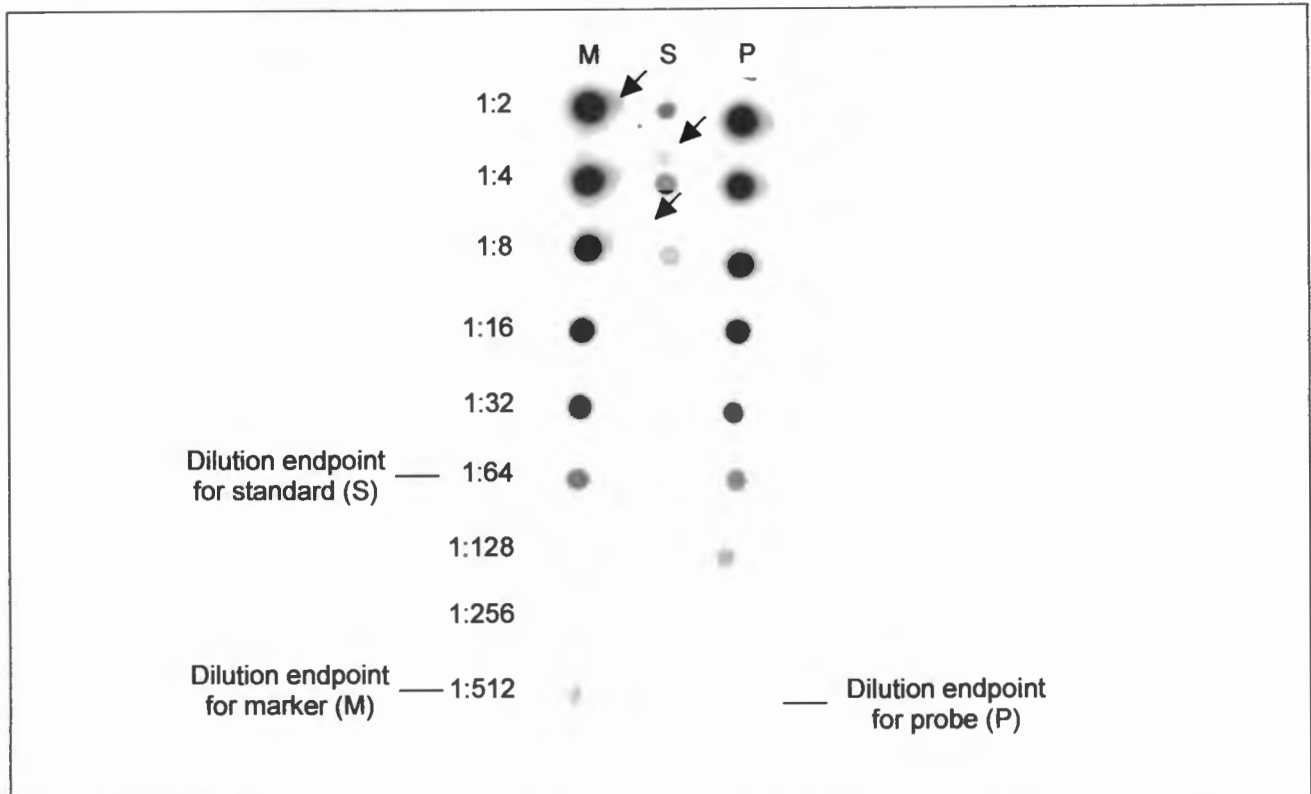


1% Agarose gel electrophoresed at 100 V in 1X TBE for 30 minutes. C = DIG labelled control DNA, M = 100 bp marker, P1 = 750 $\mu\text{g}\cdot\mu\text{l}^{-1}$ plasmid with 1:3 ratio of DIG-dUTP: dTTP, P2 = 100 $\mu\text{g}\cdot\mu\text{l}^{-1}$ plasmid with 1:3 ratio of DIG-dUTP: dTTP, P3 = 10 $\mu\text{g}\cdot\mu\text{l}^{-1}$ plasmid with 1:3 ratio of DIG-dUTP: dTTP, P4 = 10 $\mu\text{g}\cdot\mu\text{l}^{-1}$ plasmid with 1:6 ratio of DIG-dUTP: dTTP.

4.1.3.2 Biotin labelling of probe p13E-11

The biotin labelled dCTP incorporation via the random primed based method also yielded good results. The series dilution in Figure 4.7 illustrates that even the 1 in 512 dilution contained biotin incorporation. In order to determine the relative amount of biotin incorporated into the labelled MWM and p13E-11, the relative specific activity (RSA) was calculated. The first step was to determine the ratio of labelled probe to standard from the dot blot. Therefore, since the dilution endpoint, i.e. the most diluted spot visible for labelled p13E-11 and the quantitation standard was 1 in 512 and 1 in 64 respectively, an 8:1 ratio of probe concentration to standard could be determined. Thus, since the RSA of the quantitation standard is known, i.e. 50 $\text{ng}\cdot\mu\text{l}^{-1}$, the RSA of p13E-11 was calculated to be 400 $\text{ng}\cdot\mu\text{l}^{-1}$. This indicates that a successful labelling reaction was performed and that ample labelled probe was obtained for hybridisation and detection of the 4-type and 10-type fragments.

Figure 4.7: Dot blot illustrating labelling efficiency of the biotin random primed method



M = biotin labelled molecular weight marker, P = biotin labelled p13E-11, S = biotin labelled quantitation standard.

Figure 4.7 indicates that effective biotin labelling was obtained. However, there are some irregularities visible on the blot. It is clear that the MWM was not diluted with accuracy, since the 1:128 dilution spot displays no labelled probe. Given that the 1:256 and 1:512 spots both display signal, the absence of signal at the 1:64 spot was assumed to be due to dilution or experimental error. The dots indicated by the arrows occurred due to movement of the autoradiograph whilst positioning it onto the blot for the chemiluminescent detection. The endpoint dilution of the MWM, at 1:572, also displays an uneven distribution of biotin. This may have occurred due to insufficient resuspending of the dilution before spotting it on the membrane, or may be due to the presence of an artefact in the dilution mixture.

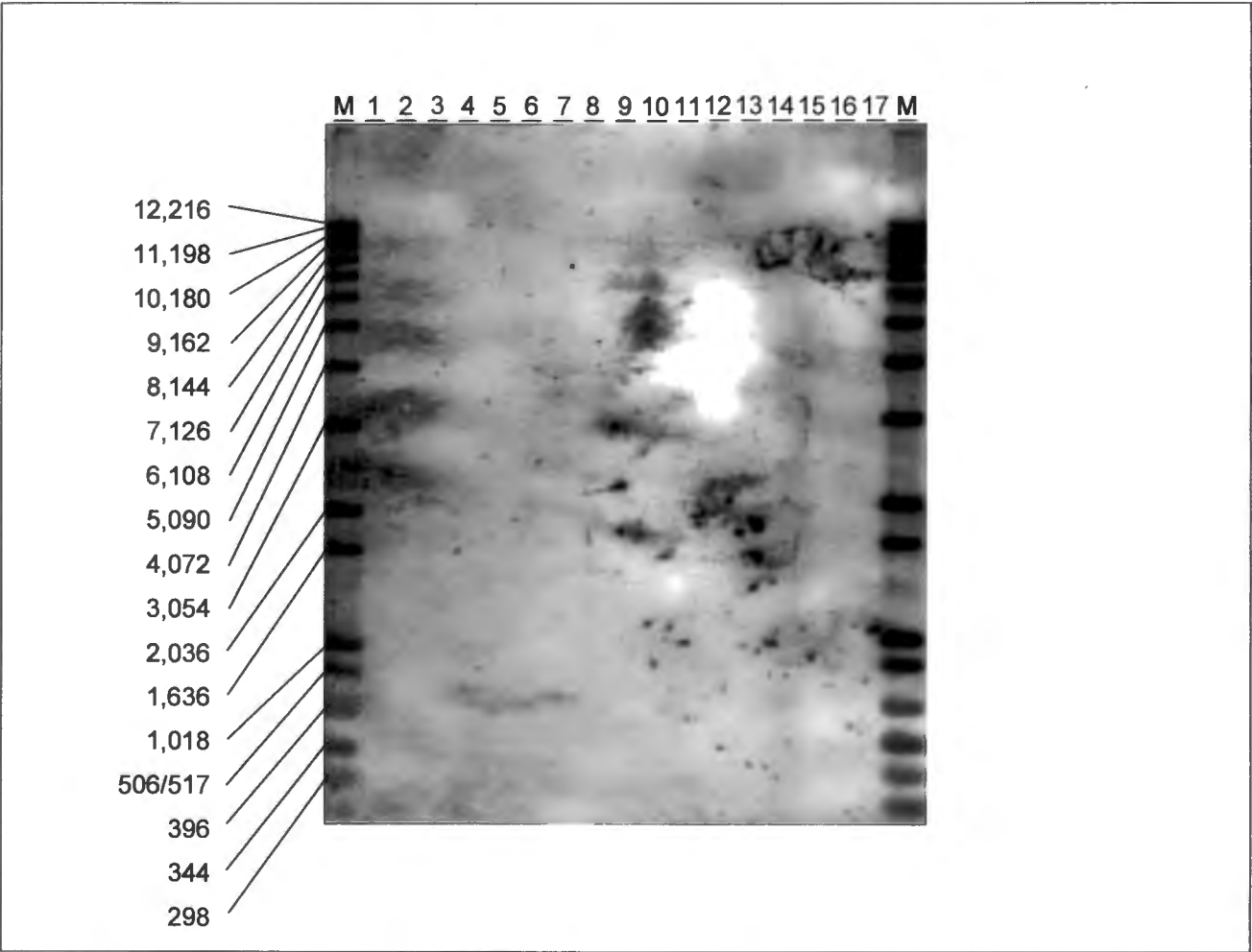
4.1.4 Hybridisation

Procedures for prehybridisation and hybridisation of the membranes depended on whether DIG-labelled or biotin labelled probes were used. DIG based protocols were initially followed utilising the specified 25 ng of probe DNA per ml of hybridisation solution and

overnight incubation at the calculated T_{hyb} of 42°C. The stringency washes and detection protocols were performed as described in sections 3.2.3.9 and 3.2.3.10.

The results revealed an autoradiograph, as presented in Figure 4.8, with a relatively high degree of background and no hybridisation of p13E-11. However, the labelled 1 kb MWM, which is 100% homologous to its target, gave a strong hybridisation signal. The MWM acts as a good positive control indicating that conditions were at least favourable for completely homologous sequence hybridisation. Detection of the MWM hybridisation also confirmed that the results obtained in section 4.1.1 were accurate and that DNA was indeed transferred from the gel and fixed to the membrane. The variation in background intensities across the blot is due to unspecific binding of the two probes to the membrane as well as to uneven distributions of CSPD during its incubation period (section 3.2.3.11). Troubleshooting and possible reasons for failure to detection of fragments via p13E-11 are discussed in section 4.1.5.

Figure 4.8: Autoradiograph exposed to DIG labelled p13E-11 and 1 kb MWM



M = DIG labelled 1kb molecular weight marker (bp), 1-17 represents the lane numbers on this blot.

4.1.5 Optimisation of detection strategies

In order to ensure that the released CSPD chemiluminescent signal was optimal, the preincubation and autoradiography periods were increased. The preincubation period of the membrane containing CSPD at 37°C was increased to two hours, as opposed to the previously suggested 10 minutes. The membrane was exposed to an autoradiograph for a series of different time periods. Results indicated that only an increase in MWM and background intensity was obtained. No hybridisation with p13E-11 in any of the samples was detected. The fact that the MWM yielded a hybridisation signal and that the signal improved with increased exposure times indicated that the chemiluminescent detection process was indeed functional. It was, therefore, assumed that the reason for failure to detect signal in the individual samples resided in an experimental procedure prior to this step.

The likelihood that gDNA was not transferred to the membrane is minimal, as there is sufficient evidence for this. Firstly, the photographic representation of the post-blot gel (Figure 4.2) indicated that there was indeed no DNA remaining in the gel after the overnight transfer was performed. Moreover, the fact that the MWM hybridised to its target verifies that there was DNA transfer.

The possibility that T_{hyb} for annealing of p13E-11 to its target was too stringent seemed likely. It may have been that the 42°C temperature was too high and prevented annealing of the probe to its target. Additional experiments were performed where T_{hyb} was decreased to 40°C and 38°C respectively. Once again the results revealed no evidence of target hybridisation and a similar blot to that presented in Figure 4.8 was obtained.

Too little probe added to the hybridisation solution may also have been the problem. The initial probe concentration in the hybridisation solution was approximately 45 ng.mL⁻¹. Subsequent experiments contained a series of probe concentrations of as high as 250 ng.mL⁻¹. However, probe concentration can only be increased up to a certain amount, after which severe background hybridisation will result. Once again no hybridisation signal was obtained, and as expected the background probe intensities increased dramatically, even with very short autoradiograph exposure times. The autoradiograph revealed a similar result as that presented in Figure 4.8.

It was suggested that the probe DNA may not have been denatured completely or may have reannealed back onto itself before it was added to the hybridisation buffer. Consequently, it was decided that when hybridisation solutions were being used more than once, to ensure that denaturation occurred at the specified 68°C for 10 minutes, and to limit the time period from denaturation of p13E-11 to placing the solution in the hybridisation bottles for incubation. Alteration of this parameter brought no improvement about.

Optimisation of Southern blot analyses is most commonly achieved by varying the stringency washes of the membrane. It was proposed that the wash stringency in previous experiments was too high and that the probe was removed from its target due to this. By altering the salt concentrations and temperature of the wash to adopt a slightly lower stringency, the probe would remain annealed to its target. However, by lowering the stringency of wash conditions, the probability of visualising more background would increase. Subsequent blots were hybridised and washed utilising lowered stringencies. This was obtained by altering both salt concentrations and incubation temperatures. Initially, the membranes were washed twice at low stringency in 2X SSC. 0.1%SDS at room temperature, followed by two high stringent washes at 65°C in 0.5X SSC. 0.1%SDS. The high stringency buffer was altered to higher salt concentrations in the order of 1X SSC and SDS to 0.5%. The temperature of the high stringency washes was decreased to 50°C. No matter what combination of SSC and SDS concentrations used, nor at what temperature the washes were performed, p13E-11 still could not be detected to its target on the membrane.

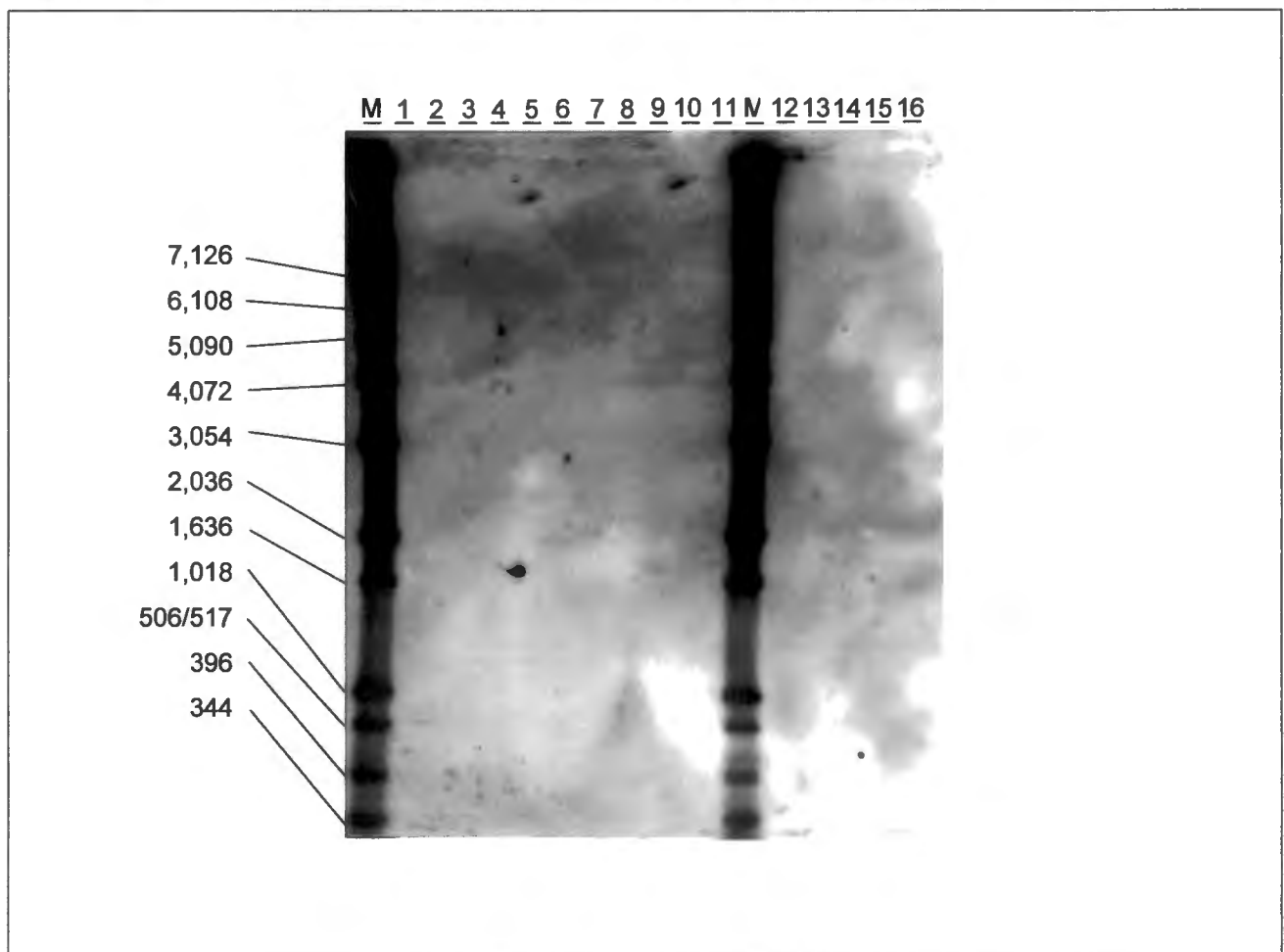
The possibility that the digested gDNA on the membrane may not have been denatured effectively enough during the transfer process was unlikely, but was given some consideration. Once again, the MWM, acting as internal positive, aided in the deduction that DNA denaturation of the MWM was obtained since probe hybridisation took place.

Finally, the concentration gDNA on the membrane was considered. The Roche Application Manual suggests that the concentration be kept low, i.e. in the order of 2.5 µg to 5 µg. The dosage test digestions were then set up in a series of 2.5 µg DNA concentrations ranging from 5 µg to 12.5 µg of gDNA. The standard protocols, as discussed in chapter, were followed with no modifications. Hybridisation of the probe was expected, depicting varied intensities corresponding to the respective gDNA

concentrations on the membrane. However, detection revealed no p13E-11 hybridisation. The blot was stripped, hybridised and detected again. This time, however, the stringency of post-hybridisation washes was decreased. Once again, p13E-11 did not hybridise.

At this point it was decided to attempt the biotin based KPL kits as discussed in chapter three. The previously used blots in the DIG based experimentation were stripped and utilised again for this phase of the experimentation process. The membranes containing 2.5 μg of digested gDNA were hybridised utilising 100 ng.ml^{-1} of biotin labelled probe p13E-11 and 50 ng.ml^{-1} of MWM. The advised stringency washes (section 3.2.3.10) and detection procedures (section 3.2.3.12) were followed. As with the DIG based procedures, no hybridisation signal was obtained for p13E-11. The labelled MWM did, however, hybridise and good detection signals were obtained.

Figure 4.9: Dosage test Southern blot utilising biotin labelled probes



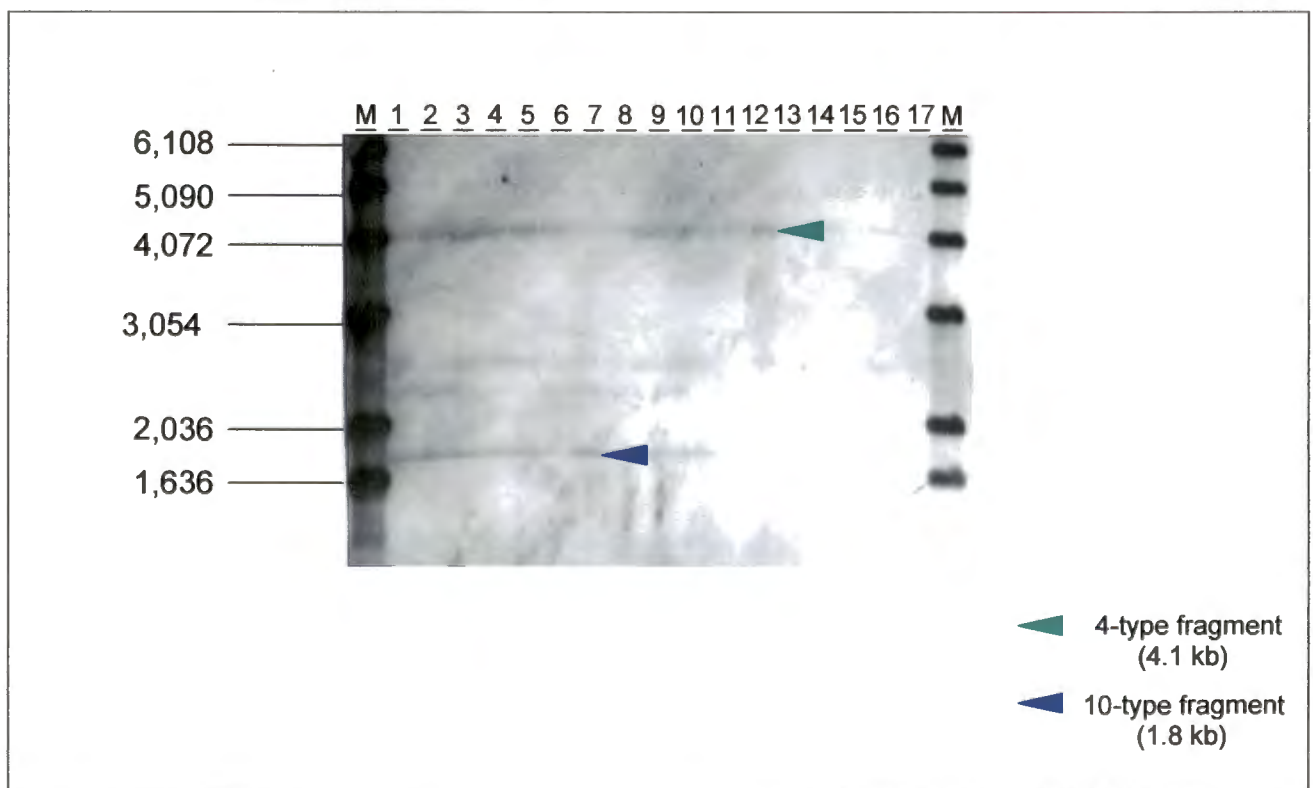
M = biotin labelled 1kb molecular weight marker (bp), 1-16 represents the lane numbers of this blot.

One of the membranes with 5 μg of fixed digested gDNA was hybridised with the previously used hybridisation solution, again containing 100 ng.ml^{-1} of biotin labelled probe

p13E-11 and 50 ng.ml⁻¹ of MWM. The same conditions were followed as for the 2.5 µg gDNA blot discussed above. The results again showed hybridisation of MWM, but no p13E-11 signal.

It was then decided to hybridise another blot with 5 µg of gDNA fixed to it, once again utilising the same hybridisation solution containing 100 ng.ml⁻¹ of biotin labelled probe p13E-11 and 50 ng.ml⁻¹ of MWM. This time, however, instead of washing the membrane in 2X SSPE/0.1%SDS at room temperature and 0.2X SSPE/0.1%SDS at 55°C for low and high stringency washes respectively, the high stringency wash was altered. The stringency was lowered by increasing the salt concentration to 1X SSPE/0.1%SDS and performing the wash at 45°C. For the first time the results indicated hybridisation of p13E-11 to the specific 4-type and 10-type fragments. Figure 4.10 presents the autoradiograph of the biotin labelled p13E-11 hybridising to fragments generated by the *Bgl* II – *Bln* I digestions. The green arrow depicts one of the 4.1 kb fragments generated by the dosage test restriction enzyme digestion, whilst the blue arrow points toward one of the fragments that originated from chromosome 10.

Figure 4.10 **Autoradiograph representation of dosage test Southern blot utilising biotin labelled probes**



M = biotin labelled molecular weight marker(bp), 1-17 represents the lane numbers of this blot.

The figure illustrates that there was still a great degree of background binding of the probes. The lower section of the gel seemed to display greater background intensity compared to the top. This may have been due to uneven exposure of the membrane to CDP-Star[®] and a subsequent higher concentration residing at the lower half of the membrane. The MWM fragments were clearly visible and allowed for easy determination of fragment sizes. It is also clear that an increased amount of CDP-Star[®] resided on the left hand side of the membrane, since the intensity of fragments of the left and right MWMs differ considerably.

Subsequent blots containing the remaining individuals investigated in the study were analysed utilising the same hybridisation conditions and stringency washes. Results were obtained showing hybridisation of p13E-11 to targets of 4-type and 10-type origin. The blots were subsequently quantified followed by data interpretation.

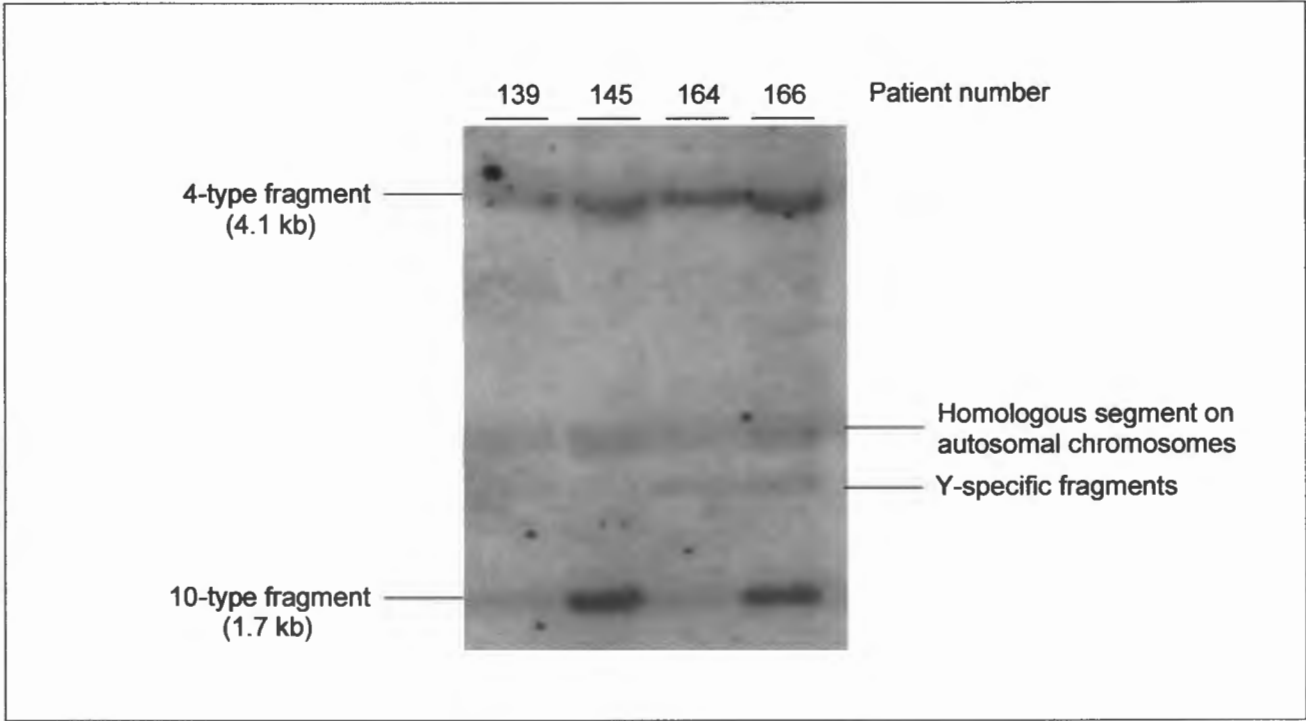
4.1.6 Quantification of detected fragments

Van der Maarel *et al.* described the dosage test in 1999 utilising a phosphorimager to directly visualise the detected fragments on the Southern blot membranes. The ImageQuant software programme [Molecular Dynamics] was utilised to calculate the intensities of the fragments. However, since an equivalent system was not available and its cost prohibitive, an alternative quantification strategy was adopted utilising the Adobe Photoshop[®] 6.0 programme. The autoradiographs of the blots containing the individuals investigated were scanned to form photo images and stored in bitmap format, followed by fragment quantification. The contrast of each fragment was determined on a scale of 1 to 100, 1 being white and 100 black. Therefore, values between 1 and 100 indicated the intensities of each of the fragments. A cursor was pointed at the site of measurement, quantifying the contrast of a specific point on the fragment. As the pointer was dragged over a fragment, readings were recorded at different points. In order to obtain an approximate (overall) intensity reading of a fragment, five contrast readings at different sites of the fragment were recorded and an average value of the signals were calculated, indicated by S_{ave} . However, the background surrounding each of the fragments contributes to the intensity of that fragment. Therefore, certain fragments may appear more or less intense depending on their backgrounds. This lack in consistency of background intensities required that contrast readings in these areas be taken into consideration. Measurements were taken from above and below each of the fragments and an average of the two readings calculated, indicated by B_{ave} . The difference of the two

average scores ($S_{ave} - B_{ave}$) was calculated in order to obtain a final or actual intensity value for each fragment. Once the true signal values were calculated, it was possible to compare fragment intensities of fragments from 4-type and 10-type origins and calculate ratios for these two readings. An example of this calculation is discussed below.

Figure 4.11 displays an excerpt of a dosage test Southern blot illustrating p13E-11 hybridisation to the chromosome 4-type and 10-type fragments. Four individuals are indicated on this blot. The autoradiograph revealed that an additional two fragments were detected by probe p13E-11, the one being of Y-chromosome origin and the other generated via hybridisation of the probe to one of the autosomes. The Y-specific fragment is present in all of the male individuals on this blot but not in individual 145, who is female. However, it may not seem clear from Figure 4.11 that this fragment is present in individual 139. This is due to loss of resolution of the scanned autoradiograph. The fragment could be observed on the original autoradiograph. The origin of the autosomal fragment is, however, unknown. Van der Maarel *et al.* (1999) did not report the presence of any other non-specific fragments, besides those from the Y-chromosome. However, from previous experience (Van der Merwe, 2002), it is known that this probe detects various other homologous regions within the human genome.

Figure 4.11: Dosage test Southern blot utilising biotin labelled probes



Visual inspection of 4-type and 10-type fragments generated from individual 139 indicates that the ratios of the fragments are relatively equal, meaning that two are of 4q origin and

the other two copies from 10q. However, a visual inspection cannot be used to quantify fragment ratios with certainty.

Table 4.1 and Table 4.2 lists the scores and fragment ratios that were generated from the analysis of individual 139. Table 4.1 first lists the five data point readings obtained from the two fragments (4.1 and 1.8 kb), indicated by S1-S5, along with the calculated S_{ave} value. Similarly, two background intensity data points are listed and the average score, B_{ave} , calculated. Finally, the signal values for the fragments are calculated via subtraction and listed below the individual signal points.

Table 4.1: Calculation of signal values for individual 139

Individual 139									
Fragment	Signal						Background		
	S1	S2	S3	S4	S5	S_{ave}	B1	B2	B_{ave}
4.1 kb	61.0	60.0	58.0	59.0	62.0	60.0	53.0	55.0	54.0
	Signal value = $S_{ave} - B_{ave} = 6.0$								
1.8 kb	55.0	57.0	56.0	54.0	55.0	55.4	49.0	51.0	50.0
	Signal value = $S_{ave} - B_{ave} = 5.4$								

Table 4.2 first lists the signal values calculated in Table 4.1, followed by the signal normalisation values. The intensity value for the 4.1 kb fragment is normalised to one, by dividing it by itself, and the 1.8 kb fragment signal normalised to that of the 4.1 kb fragment signal by division, as indicated in the table.

Table 4.2: Calculation of allele configuration for individual 139

Individual 139					
4.1 kb Fragment	1.8 kb Fragment	Normalised Signal NS4.1 (S4.1 / S4.1)	Normalised Signal NS1.8 (S1.8 / S4.1)	NS4.1/ NS1.8	Ratio of 4q: 10q
Signal (S4.1)	Signal (S1.8)				
6.00	5.40	1.00	0.90	1.11	2:2

In order to finally determine the ratio of the fragments to each other, the normalised value for the 4.1 kb fragment is divided by that of the 1.8 kb fragment. It was assumed that four fragments were under investigation, given the diploid nature of the human genome. Excluding the possibility of deletion in these regions of the genome, it was expected that

two fragments from chromosomes 4q and 10q respectively were analysed. Hence the approximate 1:1 ratio was interpreted as representing a 2:2 ratio for the four expected fragments.

The 2:2 ratio indicates that two fragments containing the *Bln* I restriction site, i.e. of 10q origin, and two fragments that do not have a restriction site for *Bln* I were generated by the *Bgl* II – *Bln* I dosage test. These results show that individual 139 is indeed disomic for the first repeat from the D4Z4 locus on chromosome 4. The majority of the individuals investigated in this study displayed equal ratios of 4-type and 10-type fragments, as will be discussed later in this section.

Individuals 145, 164 and 166 were also quantified in the same manner as described above. The allelic ratios of family members 145 and 166 are visibly more intense than those of number 139. However, taking into account the background intensity and comparative normalisations, it was evident that no irregularities in intensities existed and a disomic allelic configuration could be inferred.

The allelic distribution observed in individual 164 was slightly different to that of the other three on the blot, as presented in Figure 4.11. After visual inspection of the respective fragments it was suspected that a skewed ratio of 3:1 for chromosomes 4 to 10 may be present. Subsequent to statistical analyses and normalisation, the calculated ratios of individual 164 were confirmed. Table 4.3 lists the statistical allelic configuration of individual 164. The normalisation value of the chromosome 10-type repeat revealed an unambiguous 3:1 ratio. The intensity measurements of 4.1 kb and 1.8 kb fragments, along with their background scores are listed in Tables A.1 and A.2 of Appendix A.

Table 4.3: Calculation of allele configuration for individual 164

Individual 164					
4.1 kb Fragment	1.8 kb Fragment	Normalised Signal NS4.1 (S4.1 / S4.1)	Normalised Signal NS1.8 (S1.8 / S4.1)	NS4.1/ NS1.8	Ratio of 4q: 10q
Signal (S4.1)	Signal (S1.8)				
13.90	4.30	3.23	1.00	3.23	3:1

The remainder of individuals were quantified via the same protocol, as described above. The results and calculated ratios are presented in Table 4.4. Since the biotin based detection strategy had not been fully optimised yet, the full complement of individuals

could not be quantified. Some fragments were visible on the autoradiographs, but unfortunately less visible after scanning it for quantification. Ultimately, 69 of the 98 individuals could be accurately quantified.

Table 4.4: Ratio of fragments originating from chromosomes 4q and 10q

Patient number ¹	4.1 kb Fragment			1.8 kb Fragment			Normalised Signal NS4.1 ⁶ (S4.1 / S4.1)	Normalised Signal NS1.8 ⁶ (S1.8 / S4.1)	NS4.1 / NS1.8 ⁶	Ratio of 4q: 10q ⁷
	Signal Average (S _{ave}) ²	Background average (B _{ave}) ³	Signal (S4.1) ⁴	Signal Average (S _{ave}) ²	Background average (B _{ave}) ³	Signal (S1.8) ⁴				
75	48.0	43.0	5.00	41.6	26.0	15.60	1.00	3.12	0.32	1:3
93	45.8	41.0	4.80	39.8	24.5	15.30	1.00	3.19	0.31	1:3
206	7.4	1.0	6.40	44.0	27.5	16.50	1.00	2.58	0.39	1:3
109	30.0	22.0	8.00	38.4	14.5	23.90	1.00	2.99	0.33	1:3
164	63.4	49.5	13.90	56.8	52.5	4.30	3.23	1.00	3.23	3:1
229	14.2	4.5	9.70	26.6	23.5	3.10	3.13	1.00	3.13	3:1
24	50.6	47.0	3.60	49.4	45.5	3.90	1.00	1.08	0.92	2:2
35	51.6	45.5	6.10	54.4	48.0	6.40	1.00	1.05	0.95	2:2
37	56.4	49.0	7.40	50.2	41.5	8.70	1.00	1.18	0.85	2:2
39	58.4	52.5	5.90	50.6	45.0	5.60	1.00	0.95	1.05	2:2
40	59.6	52.5	7.10	51.2	43.0	8.20	1.00	1.15	0.87	2:2
58	52.0	45.8	6.25	53.2	45.5	7.70	1.00	1.23	0.81	2:2
60	53.2	40.0	13.20	57.8	46.5	11.30	1.00	0.86	1.17	2:2
61	47.2	39.0	8.20	49.8	43.0	6.80	1.00	0.83	1.21	2:2
65	56.0	45.5	10.50	56.0	46.5	9.50	1.00	0.90	1.11	2:2
66	55.2	47.5	7.70	55.4	46.0	9.40	1.00	1.22	0.82	2:2
67	55.6	50.0	5.60	46.2	41.0	5.20	1.00	0.93	1.08	2:2
69	56.2	50.5	5.70	45.0	40.0	5.00	1.00	0.88	1.14	2:2
70	55.0	42.5	12.50	48.0	36.0	12.00	1.00	0.96	1.04	2:2
71	47.8	30.0	17.80	35.0	15.5	19.50	1.00	1.10	0.91	2:2
76	46.2	39.0	7.20	30.4	22.0	8.40	1.00	1.17	0.86	2:2
77	47.2	42.0	5.20	28.8	23.5	5.30	1.00	1.02	0.98	2:2
80	47.6	41.0	6.60	26.6	20.5	6.10	1.00	0.92	1.08	2:2
81	45.0	38.5	6.50	26.6	21.0	5.60	1.00	0.86	1.16	2:2
85	50.0	37.5	12.50	30.2	20.5	9.70	1.00	0.78	1.29	2:2
90	41.0	29.0	12.00	32.8	19.0	13.80	1.00	1.15	0.87	2:2
91	43.8	29.0	14.80	36.0	20.0	16.00	1.00	1.08	0.93	2:2
92	47.8	38.0	9.80	35.8	24.5	11.30	1.00	1.15	0.87	2:2
95	39.6	32.0	7.60	41.6	32.0	9.60	1.00	1.26	0.79	2:2
96	46.0	34.0	12.00	44.2	34.5	9.70	1.00	0.81	1.24	2:2
97	48.8	39.0	9.80	52.0	43.5	8.50	1.00	0.87	1.15	2:2
98	55.2	46.0	9.20	54.2	46.0	8.20	1.00	0.89	1.12	2:2
99	52.0	44.0	8.00	56.6	50.0	6.60	1.00	0.83	1.21	2:2
100	54.6	45.0	9.60	50.4	40.5	9.90	1.00	1.03	0.97	2:2
101	55.2	44.5	10.70	44.6	33.0	11.60	1.00	1.08	0.92	2:2
102	56.2	42.5	13.70	42.2	27.5	14.70	1.00	1.07	0.93	2:2
103	52.0	44.5	7.50	31.6	22.6	9.00	1.00	1.20	0.83	2:2
105	47.4	36.0	11.40	34.4	24.0	10.40	1.00	0.91	1.10	2:2
106	41.0	29.5	11.50	26.6	16.0	10.60	1.00	0.92	1.08	2:2
113	43.8	26.3	17.55	22.2	7.3	14.90	1.00	0.85	1.18	2:2
116	44.6	38.5	6.10	19.0	12.3	6.70	1.00	1.10	0.91	2:2
119	66.6	63.5	3.10	53.6	49.5	4.10	1.00	1.32	0.76	2:2
120	67.8	62.5	5.30	60.2	55.0	5.20	1.00	0.98	1.02	2:2
133	48.4	46.0	2.40	52.2	50.0	2.20	1.00	0.92	1.09	2:2

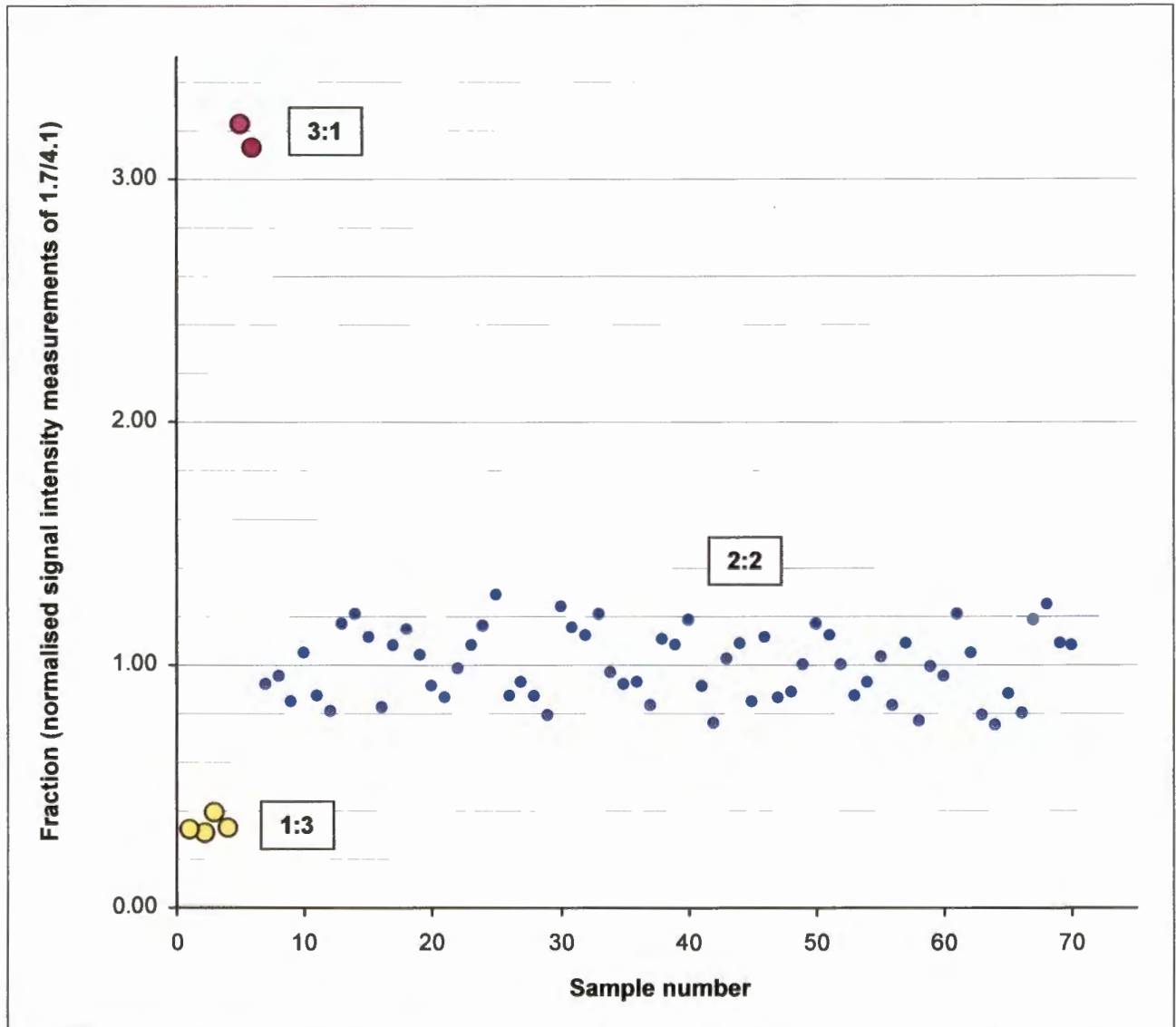
Patient number ¹	4.1 kb Fragment			1.8 kb Fragment			Normalised Signal NS4.1 ⁵ (S4.1 / S4.1)	Normalised Signal NS1.8 ⁵ (S1.8 / S4.1)	NS4.1 / NS1.8 ⁶	Ratio of 4q: 10q ⁷
	Signal Average (S _{ave}) ²	Background average (B _{ave}) ³	Signal (S4.1) ⁴	Signal Average (S _{ave}) ²	Background average (B _{ave}) ³	Signal (S1.8) ⁴				
134	49.6	43.5	6.10	54.2	47.0	7.20	1.00	1.18	0.85	2:2
139	60.0	54.0	6.00	55.4	50.0	5.40	1.00	0.90	1.11	2:2
145	60.6	51.5	9.10	65.8	55.3	10.55	1.00	1.16	0.86	2:2
166	64.4	53.0	11.40	64.8	52.0	12.80	1.00	1.12	0.89	2:2
178	55.8	49.0	6.80	53.8	47.0	6.80	1.00	1.00	1.00	2:2
183	50.8	40.0	10.80	58.2	49.0	9.20	1.00	0.85	1.17	2:2
184	48.6	41.0	7.60	53.8	47.0	6.80	1.00	0.89	1.12	2:2
187	52.8	43.0	9.80	54.8	45.0	9.80	1.00	1.00	1.00	2:2
188	47.2	35.5	11.70	59.0	45.5	13.50	1.00	1.15	0.87	2:2
189	48.0	35.5	12.50	58.4	45.0	13.40	1.00	1.07	0.93	2:2
195	56.6	49.0	7.60	63.6	56.3	7.35	1.00	0.97	1.03	2:2
205	8.2	1.0	7.20	35.2	26.5	8.70	1.00	1.21	0.83	2:2
207	13.4	4.0	9.40	47.6	39.0	8.60	1.00	0.91	1.09	2:2
211	18.8	9.0	9.80	41.2	28.5	12.70	1.00	1.30	0.77	2:2
212	33.2	19.0	14.20	40.8	26.5	14.30	1.00	1.01	0.99	2:2
213	43.0	26.5	16.50	40.8	23.5	17.30	1.00	1.05	0.95	2:2
214	38.4	23.5	14.90	34.8	22.5	12.30	1.00	0.83	1.21	2:2
220	22.4	12.5	9.90	30.4	21.0	9.40	1.00	0.95	1.05	2:2
221	10.2	6.0	4.20	23.8	18.5	5.30	1.00	1.26	0.79	2:2
222	9.6	4.0	5.60	25.0	17.5	7.50	1.00	1.34	0.75	2:2
236	45.6	25.5	20.10	44.4	21.5	22.90	1.00	1.14	0.88	2:2
402	58.4	32.5	25.90	62.2	30.0	32.20	1.00	1.24	0.80	2:2
458	52.8	35.5	17.30	54.2	39.5	14.70	1.00	0.85	1.18	2:2
459	57.2	31.5	25.70	54.6	34.0	20.60	1.00	0.80	1.25	2:2
480	50.8	22.5	28.30	56.4	30.5	25.90	1.00	0.92	1.09	2:2
481	21.8	10.0	11.80	39.4	28.5	10.90	1.00	0.92	1.08	2:2

1 = Patient numbers corresponding to the numbers indicated in Chapter 3; 2 = five values within each signal fragment were quantified and their average intensity is indicated here; 3 = two values within each signal fragment were quantified and their average intensity is indicated here; 4 = final signal values were obtained by subtraction of the respective background values from each of the signal values, for the respective fragments; 5 = the signal value for each fragment was divided by itself to obtain the normalised signal value; 6 = fraction indicating the intensity of the 4.1 kb fragment, relative to that of the 1.8 kb fragment; 7 = this ratio indicates how many copies of the four fragments originated from chromosome 4q, versus those originating from chromosome 10q.

Results illustrated that a total of six individuals out of the 69 that were quantified (8.7%) displayed skewed allelic ratios. Four were monosomic for repeat arrays originating from chromosome 4 (numbers 75, 93, 206, and 109) and the other two trisomic (numbers 164 and 229). See Table 4.4 for statistical confirmation of individuals with skewed ratios. Graph 4.1 illustrates a graphic representation of the distribution of fragment intensity ratios obtained in this study. The fragment ratios of those individuals who could be quantified with certainty are presented in three distinct clusters on the graph. The distribution of blue dots indicates the group of individuals that displayed a 2:2 ratio of 4q to 10q fragments. The yellow dots located lower on the graph represent the four individuals who were calculated to be monosomic for chromosome 4, namely family members 75, 93, 206, and

109. The purple dots refer to those individuals on the other side of the allelic spectrum, numbers 164 and 229, harbouring three copies of chromosome 4 fragments and only one copy from chromosome 10.

Graph 4.1: Distribution of signal intensity fractions for fragments originating from chromosomes 4q and 10q



Fractions were generated as described in section 4.1.6 and Appendix A. Ratios indicating 4q:10q origin of the various samples are indicated on the graph for each of the three categories of values.

4.2 FSHD FAMILIES INVESTIGATED

The six individuals who displayed monosomy and trisomy for chromosome 4 were investigated in the context of their pedigrees. Two of the six individuals, numbers 75 and 26 were from the group of randomly selected individuals who did not belong to any of the

FSHD families included in this study (see section 3.1.6), and will not be discussed. The remaining four family members belonged to families F10 and F20.

Interestingly, those who harboured the 3:1 ratio of 4-type to 10-type fragments i.e. members 164 and 229, were both anchored to family F10. Individuals 93 and 109, who were monosomic for chromosome 4, belonged to family F20. Whether this is due to chance or if there is a predisposing factor to translocations within these families is not known. Even more interesting is that all four the individuals harbour corresponding deletion fragments, as presented in Table 4.5 (A. Van der Merwe, 2002). The fact that the skewed ratios are complemented by corresponding deletion fragments, may strengthen the argument for a possible mutation mechanism that incorporates chromosome 10 together with other underlying factors; a scenario currently unknown in the pathogenesis of FSHD.

Table 4.5: Comparison of 4q:10q ratios with 4q35 deletion fragment sizes in four individuals

Patient number	Family	4:10 ratio	Deletion fragment ¹
93	F20	1:3	34 kb
109	F20	1:3	34 kb
164	F10	3:1	24 kb
229	F10	3:1	24 kb

¹ = Data generated by A. Van der Merwe (2002).

However, before any conclusions can be drawn, it would be imperative to investigate a larger population to determine if the monosomy or trisomy for chromosome 4 is in fact observed with a corresponding deletion fragment throughout these respective families.

CHAPTER FIVE

CONCLUSION

FSHD is a clinically and genetically heterogeneous disorder that preferentially affects the facial, shoulder girdle and upper-arm muscles (Padberg, 1982). The manifestation of the disorder is due to a reduction in allele size at the D4Z4 locus on chromosome 4q35 (Wijmenga *et al.*, 1992). The region does, however, display high distribution homology to chromosome 10q26 (Deidda *et al.*, 1995). This arrangement has been proposed in the past to contribute to and serve as a model for explaining translocation events between the two above mentioned regions (Van Overveld *et al.*, 2000). The study presented here investigated the presence of plasticity between these homologous regions on chromosomes 4q35 and 10q26.

Selected individuals from a cohort of South African families were investigated in this study. However, out of the total of 90 individuals investigated, only 69 could be genotyped with certainty. Previously, studies were based on haplotype analysis and Southern blot analysis investigating the presence of, and verification of, segregation of deletion fragments (Van der Merwe, 2002). This study, however, had the goal of investigating the presence of translocation events between the 4-type and 10-type repeat arrays, making it the first study of this kind in the South African population.

The dosage test (Van der Maarel *et al.*, 1999) was designed to exploit unique differences within the repeat units originating from chromosomes 4 and 10 respectively. Restriction endonuclease digestion utilising enzymes *Bgl* II and *Bln* I enables this distinction, as discussed in section 2.1.2.3. To date, non-radioactive detection of the 4-type and 10-type fragments, using probe p13E-11, have not been reported mainly due to the concept that only radio-labelling protocols are sensitive enough to be effective in this type of analyses. Consequently, one of the specific aims of this study was to optimise a non-radioactive detection strategy. Both biotin and DIG based procedures were investigated.

The optimisation of non-radioactive detection procedures for both DIG and biotin based methods was a challenging endeavour. Several combinations of procedural variants were attempted. Ultimately hybridisation and detection success of only biotin labelled p13E-11

was achieved. DIG detection could to date not be optimised. However, one protocol configuration that was not experimentally executed, but could have yielded successful results, was to employ the successful wash conditions (obtained with the biotin-based strategy) also for the DIG strategy. This is certainly one of the next experiments that will be executed in this ongoing research programme. An informative study in the future would be to optimise the two non-radioactive methodologies and simultaneously compare sensitivity to that of radioactive detection.

Ninety individuals were investigated in this study, from whom a total of 69 could be genotyped with accuracy. A large proportion of individuals, 91.3%, were demonstrated to have a 2:2 ratio of 4-type to 10-type fragments. Only 8.7% harboured skewed ratios of either 4-type on 10, or alternatively 10-type on 4 alleles. This is considerably less than what was expected and previously reported (Van der Maarel *et al.*, and Matsumura *et al.*, 2002). However, since a relatively small sample was investigated, it would be unrealistic to infer a frequency for translocation in the South African FSHD population. Moreover, since random individuals were selected from the family pedigrees, no clear link or consistency between members harbouring deletion fragments with corresponding occurrences of translocation events can be assumed.

The study presented does, however, illustrate that there is indeed scope for further research in this direction, not only with regards to the link between deletion fragment and skewed ratio inheritance, but also regarding the improved detection strategies that eliminate the complications associated with radioactive based detection strategies. This non-radioactive protocol improves the turnaround time for FSHD diagnosis, from 3 to 7 days via the radiolabelled protocol, to 2 to 3 days. Unlocking the potential of molecular technologies such as this holds great promise for improvement of diagnostic and treatment protocols for FSHD in the future.

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APPENDIX A

SIGNAL INTENSITY MEASUREMENTS

Signal intensities were determined as described in section 4.1.6. Five data points were identified for each of the fragments, and signal intensities were measured at these five points via Adobe Photoshop®¹. Two background intensity measurements were obtained for each fragment. The average signal and background intensities were calculated (see Tables A.1 and A.2). In each case, the background reading was subtracted from the signal reading to obtain the actual signal measurement. Normalised signal values for the 4.1 kb fragment were obtained by dividing the 4.1 kb fragment signal intensities with their numeric equivalents. Normalised signal values for the 1.8 kb fragment were obtained by dividing the 1.8 kb fragments' intensity value with that of the 4.1 kb fragment. This provided the fraction indicating the intensity of the 4.1 kb fragment relative to the intensity of the 1.8 kb fragment. The ratio of fragments from chromosomes 4q and 10q was determined when the previously obtained fraction was interpreted according to the criteria discussed in section 4.1.6.

Table A.1: Intensities measurements for the 4.1 kb fragment

Patient number	Signal						Background		
	S1	S2	S3	S4	S5	S4.1 _{ave}	B1	B2	B4.1 _{ave}
24	52.0	51.0	49.0	50.0	51.0	50.6	48.0	46.0	47.0
35	51.0	53.0	51.0	51.0	52.0	51.6	46.0	45.0	45.5
37	55.0	56.0	58.0	57.0	56.0	56.4	49.0	49.0	49.0
39	60.0	58.0	57.0	59.0	58.0	58.4	51.0	54.0	52.5
40	60.0	61.0	58.0	59.0	60.0	59.6	53.0	52.0	52.5
58	52.0	53.0	50.0	51.0	54.0	52.0	46.0	45.5	45.8
60	51.0	55.0	53.0	54.0	53.0	53.2	45.0	35.0	40.0
61	45.0	46.0	47.0	51.0	47.0	47.2	43.0	35.0	39.0
65	55.0	56.0	58.0	54.0	57.0	56.0	50.0	41.0	45.5
66	55.0	54.0	56.0	57.0	54.0	55.2	50.0	45.0	47.5
67	55.0	54.0	56.0	57.0	56.0	55.6	51.0	49.0	50.0
69	56.0	57.0	55.0	57.0	56.0	56.2	52.0	49.0	50.5
70	55.0	54.0	55.0	56.0	55.0	55.0	40.0	45.0	42.5
71	50.0	48.0	49.0	47.0	45.0	47.8	25.0	35.0	30.0
76	49.0	47.0	46.0	44.0	45.0	46.2	40.0	38.0	39.0
77	48.0	45.0	47.0	46.0	50.0	47.2	44.0	40.0	42.0
80	48.0	47.0	48.0	49.0	46.0	47.6	42.0	40.0	41.0
81	49.0	44.0	45.0	44.0	43.0	45.0	38.0	39.0	38.5
85	49.0	50.0	51.0	48.0	52.0	50.0	37.0	38.0	37.5
90	41.0	43.0	38.0	41.0	42.0	41.0	28.0	30.0	29.0
91	48.0	45.0	42.0	41.0	43.0	43.8	28.0	30.0	29.0
92	47.0	45.0	49.0	50.0	48.0	47.8	37.0	39.0	38.0
95	36.0	39.0	41.0	40.0	42.0	39.6	32.0	32.0	32.0
96	44.0	46.0	47.0	47.0	46.0	46.0	36.0	32.0	34.0
97	48.0	51.0	47.0	50.0	48.0	48.8	41.0	37.0	39.0
98	56.0	55.0	58.0	54.0	53.0	55.2	49.0	43.0	46.0
99	51.0	54.0	52.0	50.0	53.0	52.0	40.0	48.0	44.0
100	54.0	55.0	53.0	58.0	53.0	54.6	46.0	44.0	45.0
101	53.0	56.0	54.0	55.0	58.0	55.2	43.0	46.0	44.5

¹ Adobe logo and Photoshop is a trademark of Adobe systems

Patient number	Signal						Background		
	S1	S2	S3	S4	S5	S4.1 _{ave}	B1	B2	B4.1 _{ave}
102	56.0	55.0	58.0	57.0	55.0	56.2	38.0	47.0	42.5
103	54.0	53.0	51.0	52.0	50.0	52.0	46.0	43.0	44.5
105	47.0	50.0	46.0	47.0	47.0	47.4	32.0	40.0	36.0
106	40.0	43.0	40.0	40.0	42.0	41.0	33.0	26.0	29.5
113	45.0	43.0	44.0	43.0	44.0	43.8	29.5	23.0	26.3
116	43.0	43.0	44.0	47.0	46.0	44.6	39.0	38.0	38.5
119	67.0	66.0	67.0	67.0	66.0	66.6	63.0	64.0	63.5
120	68.0	68.0	67.0	69.0	67.0	67.8	62.0	63.0	62.5
133	49.0	48.0	50.0	47.0	48.0	48.4	46.0	46.0	46.0
134	51.0	48.0	49.0	50.0	50.0	49.6	42.0	45.0	43.5
139	61.0	60.0	58.0	59.0	62.0	60.0	53.0	55.0	54.0
145	60.0	61.0	62.0	59.0	61.0	60.6	51.5	51.5	51.5
166	62.0	64.0	65.0	65.0	66.0	64.4	52.0	54.0	53.0
178	55.0	54.0	56.0	56.0	58.0	55.8	47.0	51.0	49.0
183	51.0	52.0	50.0	49.0	52.0	50.8	41.0	39.0	40.0
184	48.0	51.0	47.0	50.0	47.0	48.6	42.0	40.0	41.0
187	49.0	53.0	56.0	52.0	54.0	52.8	45.0	41.0	43.0
188	46.0	46.0	49.0	47.0	48.0	47.2	33.0	38.0	35.5
189	50.0	48.0	47.0	46.0	49.0	48.0	35.0	36.0	35.5
195	57.0	56.0	55.0	58.0	57.0	56.6	49.0	49.0	49.0
205	12.0	6.0	7.0	7.0	9.0	8.2	1.0	1.0	1.0
207	12.0	15.0	11.0	15.0	14.0	13.4	4.0	4.0	4.0
211	20.0	18.0	17.0	20.0	19.0	18.8	8.0	10.0	9.0
212	32.0	34.0	35.0	33.0	32.0	33.2	20.0	18.0	19.0
213	43.0	43.0	44.0	42.0	43.0	43.0	27.0	26.0	26.5
214	40.0	38.0	40.0	36.0	38.0	38.4	29.0	18.0	23.5
220	21.0	24.0	25.0	19.0	23.0	22.4	15.0	10.0	12.5
221	10.0	11.0	8.0	12.0	10.0	10.2	4.0	8.0	6.0
222	8.0	10.0	11.0	9.0	10.0	9.6	2.0	6.0	4.0
236	45.0	47.0	46.0	44.0	46.0	45.6	24.0	27.0	25.5
402	60.0	57.0	58.0	59.0	58.0	58.4	30.0	35.0	32.5
458	50.0	54.0	55.0	52.0	53.0	52.8	35.0	36.0	35.5
459	59.0	57.0	56.0	60.0	54.0	57.2	30.0	33.0	31.5
480	53.0	52.0	51.0	47.0	51.0	50.8	21.0	24.0	22.5
481	21.0	22.0	24.0	22.0	20.0	21.8	7.0	13.0	10.0

S1 to S5 = signal intensity measurements at five data points within the 4.1 kb fragment; S_{ave} = average signal intensity [Σ (S1, S2, S3, S4, S5)/5]. B1 to B2 = background intensity measurements at two data points around the 4.1 kb fragment; B_{ave} = average background intensity [Σ (B1, B2)/2].

Signal intensities measurements for the 1.8 kb fragment are listed in Table A.2.

Table A.2: Intensities measurements for the 1.8 kb fragment

Patient number	Signal						Background		
	S1	S2	S3	S4	S5	S1.8 _{ave}	B1	B2	B1.8 _{ave}
24	49.0	51.0	49.0	50.0	48.0	49.4	45.0	46.0	45.5
35	57.0	53.0	55.0	54.0	53.0	54.4	49.0	47.0	48.0
37	51.0	52.0	48.0	49.0	51.0	50.2	44.0	39.0	41.5
39	52.0	51.0	49.0	50.0	51.0	50.6	47.0	43.0	45.0
40	53.0	50.0	52.0	51.0	50.0	51.2	48.0	38.0	43.0
58	53.0	54.0	52.0	53.0	54.0	53.2	44.0	47.0	45.5
60	57.0	58.0	59.0	58.0	57.0	57.8	46.0	47.0	46.5
61	52.0	51.0	48.0	49.0	49.0	49.8	42.0	44.0	43.0
65	57.0	56.0	55.0	57.0	55.0	56.0	45.0	48.0	46.5
66	57.0	55.0	56.0	55.0	54.0	55.4	45.0	47.0	46.0
67	47.0	45.0	48.0	45.0	46.0	46.2	42.0	40.0	41.0
69	45.0	47.0	44.0	45.0	44.0	45.0	39.0	41.0	40.0
70	47.0	49.0	47.0	48.0	49.0	48.0	37.0	35.0	36.0

Patient number	Signal						Background		
	S1	S2	S3	S4	S5	S1.8 _{ave}	B1	B2	B1.8 _{ave}
71	34.0	36.0	35.0	32.0	38.0	35.0	12.0	19.0	15.5
76	30.0	34.0	28.0	32.0	28.0	30.4	19.0	25.0	22.0
77	31.0	30.0	28.0	28.0	27.0	28.8	23.0	24.0	23.5
80	25.0	26.0	28.0	25.0	29.0	26.6	20.0	21.0	20.5
81	26.0	24.0	22.0	32.0	29.0	26.6	20.0	22.0	21.0
85	30.0	33.0	26.0	29.0	33.0	30.2	20.0	21.0	20.5
90	35.0	31.0	34.0	30.0	34.0	32.8	20.0	18.0	19.0
91	34.0	40.0	32.0	35.0	39.0	36.0	19.0	21.0	20.0
92	37.0	32.0	33.0	38.0	39.0	35.8	24.0	25.0	24.5
95	41.0	44.0	40.0	41.0	42.0	41.6	30.0	34.0	32.0
96	43.0	44.0	46.0	43.0	45.0	44.2	36.0	33.0	34.5
97	52.0	53.0	49.0	51.0	55.0	52.0	43.0	44.0	43.5
98	53.0	54.0	55.0	56.0	53.0	54.2	44.0	48.0	46.0
99	57.0	55.0	56.0	57.0	58.0	56.6	49.0	51.0	50.0
100	48.0	52.0	50.0	53.0	49.0	50.4	42.0	39.0	40.5
101	41.0	45.0	49.0	42.0	46.0	44.6	30.0	36.0	33.0
102	41.0	40.0	45.0	43.0	42.0	42.2	30.0	25.0	27.5
103	32.0	31.0	32.0	31.0	32.0	31.6	23.6	21.6	22.6
105	34.0	35.0	33.0	34.0	36.0	34.4	23.0	25.0	24.0
106	26.0	27.0	28.0	25.0	27.0	26.6	14.0	18.0	16.0
113	27.0	21.0	24.0	18.0	21.0	22.2	8.3	6.3	7.3
116	16.0	20.0	21.0	21.0	17.0	19.0	12.3	12.3	12.3
119	53.0	54.0	53.0	54.0	54.0	53.6	49.0	50.0	49.5
120	61.0	59.0	60.0	61.0	60.0	60.2	54.0	56.0	55.0
133	53.0	52.0	52.0	53.0	51.0	52.2	50.0	50.0	50.0
134	57.0	54.0	53.0	53.0	54.0	54.2	46.0	48.0	47.0
139	55.0	57.0	56.0	54.0	55.0	55.4	49.0	51.0	50.0
145	65.0	66.0	66.0	65.0	67.0	65.8	55.3	55.3	55.3
166	64.0	65.0	66.0	65.0	64.0	64.8	54.0	50.0	52.0
178	53.0	54.0	54.0	55.0	53.0	53.8	46.0	48.0	47.0
183	60.0	57.0	59.0	57.0	58.0	58.2	46.0	52.0	49.0
184	55.0	53.0	54.0	54.0	53.0	53.8	50.0	44.0	47.0
187	55.0	57.0	53.0	54.0	55.0	54.8	46.0	44.0	45.0
188	58.0	61.0	59.0	57.0	60.0	59.0	45.0	46.0	45.5
189	57.0	60.0	58.0	61.0	56.0	58.4	43.0	47.0	45.0
195	65.0	63.0	62.0	65.0	63.0	63.6	56.3	56.3	56.3
205	35.0	39.0	36.0	35.0	31.0	35.2	26.0	27.0	26.5
207	48.0	47.0	47.0	50.0	46.0	47.6	41.0	37.0	39.0
211	43.0	44.0	41.0	40.0	38.0	41.2	33.0	24.0	28.5
212	41.0	43.0	40.0	38.0	42.0	40.8	33.0	20.0	26.5
213	41.0	36.0	44.0	45.0	38.0	40.8	26.0	21.0	23.5
214	32.0	36.0	34.0	37.0	35.0	34.8	24.0	21.0	22.5
220	32.0	31.0	29.0	31.0	29.0	30.4	17.0	25.0	21.0
221	25.0	23.0	24.0	23.0	24.0	23.8	16.0	21.0	18.5
222	24.0	23.0	25.0	27.0	26.0	25.0	18.0	17.0	17.5
236	43.0	44.0	45.0	47.0	43.0	44.4	23.0	20.0	21.5
402	63.0	63.0	62.0	61.0	62.0	62.2	33.0	27.0	30.0
458	53.0	53.0	55.0	56.0	54.0	54.2	38.0	41.0	39.5
459	54.0	53.0	56.0	55.0	55.0	54.6	36.0	32.0	34.0
480	59.0	56.0	55.0	54.0	58.0	56.4	29.0	32.0	30.5
481	38.0	41.0	38.0	39.0	41.0	39.4	29.0	28.0	28.5

S1 to S5 = signal intensity measurements at five data points within the 4.1 kb fragment; S_{ave} = average signal intensity [Σ (S1, S2, S3, S4, S5)/5]. B1 to B2 = background intensity measurements at two data points around the 4.1 kb fragment; B_{ave} = average background intensity [Σ (B1, B2)/2].