1994

Localisation and detection of a polymorphism in the human skeletal Beta-Tropomyosin gene (TPM2)

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LOCALISATION AND DETECTION OF A POLYMORPHISM IN THE HUMAN SKELETAL β-TROPOMYOSIN GENE (TPM2)

A Report Submitted in Partial Fulfilment of the Requirements for the Award of
Bachelor of Applied Science
with Honours
(Biological Sciences)

by

Clive C. J. Hunt

Department of Biological Sciences
Edith Cowan University

1994

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

ACKNOWLEDGMENTS

This has been a great year, where I've had an introduction to the world of science and molecular biology. For this, there are a number of people I'd like to thank.

I'd like to thank Chris Meredith for his willingness to be my supervisor this year and for all the "late night" encouragement and support he has given me through the project. You've been a great friend.

I'd also like to thank my co-supervisor Dr Nigel Laing for the privilege and opportunity it has been for me to undertake my research in collaboration with the Australian Neuromuscular Research Institute. Thank you for your enthusiasm and direction with the project.

To (nearly Dr) Anthony Akkari, I'd like to thank you for showing me most of the techniques I've used this year and for being a great friend. Life would have been duller in the long hours without your presence. Shud shonorugulem.

Thanks to Dr Steve Wilton for your help and assistance with my project.

Thanks to Bernadette for all our past midnight conversations and encouragement this year. I wish you all the best in the future. My stomach also greatly appreciated Bern's Granny, for her weekly doses of extra high calorie food. Hmmm... Granny food!

Thanks to Jo for your friendship and help throughout the year.

I'd also like to thank the Department of Cytogenetics and Molecular Genetics at Adelaide Children's Hospital for their work with FISH in localising TPM2 and using the CEPH families, to link the TPM2 polymorphism.

Also to all my scaly mates at ANRI; Lyn, Howard, Danielle, Dania and Sue, I wish you all the best.

I'd also like to thank my family, and especially my father and mother Bob and Jan Hunt for their love and constant wisdom and advice.

Finally, and mostly, I'd like to thank God and my Lord and Saviour Jesus Christ for making this all possible.

DECLARATION

"I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text."

ABSTRACT

Tropomyosin is one of the components of the thin filaments of muscle, binding to actin, and, together with troponin, regulating contraction in a calcium-dependent manner (Cho et al.,1990). There are at least four distinct tropomyosin genes in vertebrates and each may encode at least six different isoforms of tropomyosin by alternate splicing (Novy et al., 1993; MacLeod et al., 1988). The alpha-tropomyosin gene TPM1 has recently been localised to 15q22 (Eyre et al., 1994) and has been shown to be mutated in some cases of familial hypertrophic cardiomyopathy (Thierfelder et al., 1994). The alpha-tropomyosin gene TPM3 has been recently localised to 1q22-q23 (Wilton et al., 1994) and has been shown to be mutated in a family with autosomal dominant nemaline myopathy (Laing, 1994, unpublished observations).

Each muscle-specific gene is possibly associated with an inherited muscle disease, if there is a disease causing mutation in the gene. Precise mapping of muscle genes therefore becomes important in relation to mapping muscle diseases (Eyre et al., 1993).

A sequence tagged site (STS) (Olson et al., 1989) was developed for the human beta tropomyosin gene (TPM2). The STS was used to amplify DNA from somatic cell hybrids to localise TPM2 to human chromosome 9. Genomic clones isolated with the STS product were in turn used in fluorescent *in situ* hybridisation (Callen et al., 1992) to metaphase chromosome spreads to further localise TPM2 to 9p13.1 (Hunt et al., 1995).

This project should assist those laboratories searching for candidate genes of inherited muscular diseases that are linked within the region of the TPM2 gene and may assist in the precise diagnosis of people with these diseases (Akkari, 1994). Localising TPM2 also lays a foundation for a better understanding of the role of tropomyosins in muscle and nonmuscle cells.

A polymorphism was also discovered in the 3'UTR of TPM2, using single stranded conformation analysis (SSCA). The primers used were 5'-AAGTCTATGCCAGAAGATG-3' and the complementary strand 5'-CCGTGACCGAAGTAGGAAAT-3' creating a 259 bp sequence tagged site (STS). Sequencing of the STS revealed that there were two variations of the 11th base in the 3'UTR: a guanine and an adenine. Genomic DNA from 97 unrelated individuals was screened by SSCA and the allelic frequency was determined to be for the common allele (guanine) 0.91 and the rare allele (adenine) 0.09. The heterozygosity was 0.16. There is a *Bgl* I restriction site at the common allele polymorphic location. A *Bgl* I restriction digest of the 259 bp STS produces two fragments (88 & 171 bp) for homozygous individuals with the common allele, three fragments (88, 171 and 259 bp) for heterozygous individuals and a single fragment (259 bp) for homozygous individual with the rare allele.

The discovery of this polymorphism will be entered on the CEPH map and is useful for researchers as a linkage and physical marker in the human genome project, especially since the TPM2 gene has now been localised to 9p13.1.

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LIST OF ABBREVIATIONS

3'UTR	3 Prime Untranslated Region
(α-^{32}P)dCTP	Radioactive isotope of phosphorus in the alpha position in deoxycytosine triphosphate
ABI	Applied Biosystems Incorporated
ANRI	Australian Neuromuscular Research Institute
bp	Nucleotide Base Pairs
BSA	Bovine Serum Albumin
CCD	Charged Coupled Device
cDNA	Complementary Deoxyribonucleic Acid
CEPH	Centre d'Etude du Polymorphisme Humain
cpm	Counts Per Minute
DAPI	4', 6-diamidino-2-phenylimdole
ddATP	Dideoxyadenine Triphosphate
ddCTP	Dideoxycytosine Triphosphate
ddGTP	Dideoxyguanine Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
ddTTP	Dideoxythymine Triphosphate
DNA	Deoxyribonucleic Acid
dNTP's	Deoxynucleotide Triphosphate
dsDNA	Double Stranded Deoxyribonucleic Acid
ECU	Edith Cowan University
EMBL3 SP6/T7	Vector for human genomic library

EtBr	Ethidium Bromide
FISH	Fluorescence *in situ* hybridisation
hrs	Hours
kb	DNA nucleotide kilobases
LMPAG	Low Melting Point Agarose Gel
min	Minutes
mRNA	Messenger Ribose Nucleic Acid
NHMRC	National Health & Medical Research Council
PCR	Polymerase Chain Reaction
pfu	Plaque Forming Units
PNK	Polynucleotide Kinase End Labelling
RNA	Ribose Nucleic Acid
rpm	Revolutions per Minute
sk	Muscle Specific Exon only Spliced with Muscle Isoforms
SSCA	Single Stranded Conformation Analysis
SSCP	Single Stranded Conformation Polymorphism
ssDNA	Single Stranded Deoxyribonucleic Acid
STS	Sequence Tagged Site
T tubule	Transverse tubule
TPM	Tropomyosin
TPM2	Tropomyosin 2 gene
UV	Ultraviolet light
V	Volts

ERRATA

Figure 7.2 on p. 126 should read ~250 bp of the STS size, instead of 500 bp.

CHAPTER 1

INTRODUCTION

1.1 THE TROPOMYOSIN PROTEIN

Tropomyosin (TPM) is an important component of the contractile unit in skeletal muscle, playing a pivotal role in regulating the interaction between actin and myosin filaments (Gunning, et al., 1990). Tropomyosins are rod-like, actin-binding proteins found in most muscle and nonmuscle tissues. In muscle, tropomyosin's structure is two alpha-helical chains (except for the extreme ends) wound around the long pitch grooves of the helical actin filament, in a coiled-coil structure, head to tail (see Figure 1.1) (Wolfe, 1993). Muscle tropomyosin is 284 amino acids long, spans seven actin monomers and makes seven half-turns per molecular length. In nonmuscle cells tropomyosins are associated with the actin-containing microfilaments of the cytoskeleton (MacLeod, et al., 1987). Certain non-muscle tropomyosins, the best studied being platelet, are shorter, only 247 amino acids in length and have a length corresponding to six half-turns per molecule and six instead of seven actin monomers (Hitchcock-DeGregori & Varnell, 1990). Different isoforms of tropomyosin have been described, though a specific function for the protein is best defined in skeletal muscle (Cho, et al., 1990; MacLeod & Gooding, 1988).

1.2 TROPOMYOSIN'S ROLE IN MUSCLE CONTRACTION

Striated muscle contraction is regulated by Ca^{2+} through the troponin-tropomyosin complex (see Figure 1.2). For muscles at rest, tropomyosin normally blocks all the actin binding sites with myosin on the actin microfilaments. The troponin subunits TnI and TnT normally bind to tropomyosin holding it in the myosin blocking position (a&c). When a nerve impulse causes a voltage change in a muscle cell plasma membrane, the

FIGURE 1.1

Tropomyosin and its Association with Troponin.

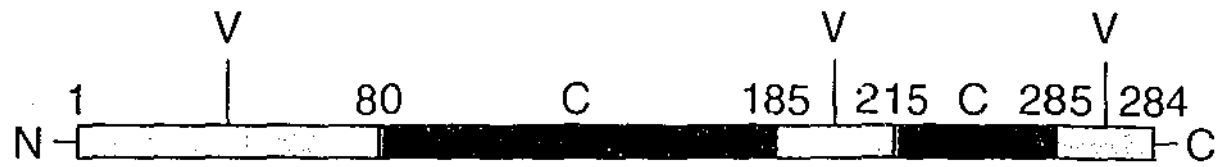
(a) The coiled-coil structure of Tropomyosin

(b) Sequence structure of striated and smooth forms of tropomyosin, showing the locations in which the amino acid sequence is conserved (C) and variable (V) among different tropomyosin types. Nonmuscle tropomyosins are shorter in length but have conserved segments at equivalent distances from the C-terminal end.

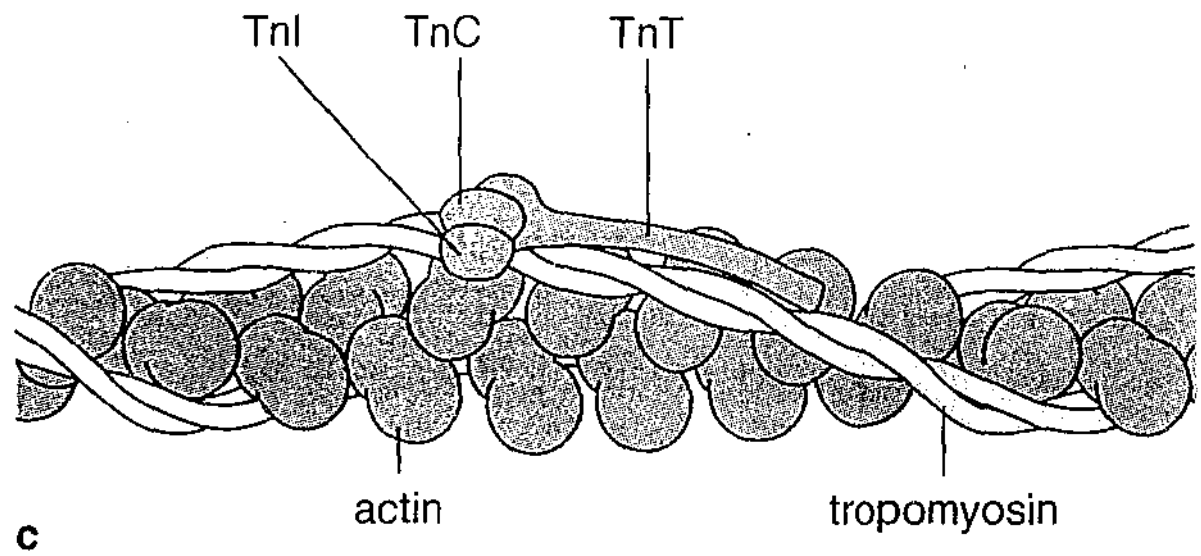
(c) The arrangement of tropomyosin and the TnC, TnI and TnT subunits of troponin on striated muscle microfilaments.

(After Wolfe, 1993)

a tropomyosin



b



c

FIGURE 1.2

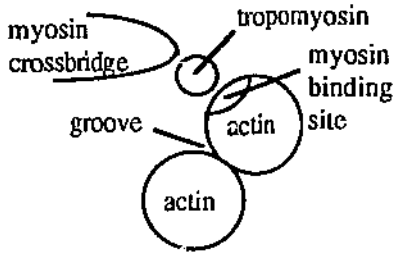
Calcium Ion Regulation in Muscle Contraction

The actin-linked control mechanism involving blockage by tropomyosin of the crossbridge binding sites on microfilaments, as seen in a cross section of a microfilament.

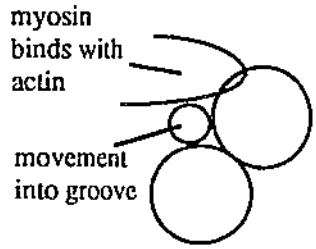
(a) In the blocking position, tropomyosin covers binding sites for myosin crossbridges.

(b) Movement of tropomyosin toward the microfilament groove exposes the myosin-binding sites and triggers the crossbridging cycle **(c)** and **(d).**

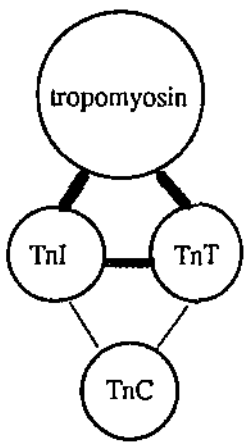
(After Wolfe, 1993)



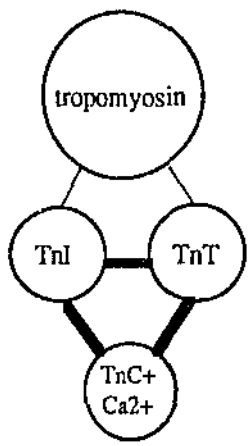
a - when low [Ca2+] tropomyosin blocks myosin-actin interaction



b - when high [Ca2+] tropomyosin moves into actin groove



c - tropomyosin binds with TnI and TnT, in blocking position, when low [Ca2+]



d - when released Ca2+ binds TnC, the tropomyosin and TnI and TnT bonds break

impulse travels via the transverse tubule (T tubule) to the sarcoplasmic reticulum and causes a release in Ca^{2+} from the Ca^{2+} -ATPase pump. The Ca^{2+} binds with the third troponin subunit, TnC, and it undergoes conformational changes and increases its affinity to TnI and TnT, weakening the tropomyosin and TnI and TnT bonds (b&d). Tropomyosin then moves towards the actin microfilament groove exposing the myosin binding sites, allowing a series of processes so the muscle contracts (Borovikov, et al., 1993; MacLeod & Gooding, 1988; Wolfe, 1993).

Hitchcock-DeGregori and Varnelli, (1990) have experimentally shown that muscle tropomyosin has periodic binding sites with actin in two sets of seven sites. This corresponds to the seven actin monomers it spans and the two states it exists in, ie. either muscle contraction or rest. One set of seven sites function in actin binding and both sets of seven function in the calcium regulation via troponin of muscle contraction (Hitchcock-DeGregori & Varnell, 1990).

1.3 THE TROPOMYOSIN GENES AND ALTERNATIVE SPLICING OF DIFFERENT ISOFORMS

There are at least four separate, non overlapping tropomyosin genes, that encode at least six different isoforms of tropomyosin in muscle and non-muscle human fibroblasts (see Table 1) (Clayton, et al., 1988; Gunning, et al., 1990). Three of the four tropomyosin genes have been sequenced from expressed mRNA encoding the human skeletal muscle isoforms, TPM1 (MacLeod & Gooding, 1988), TPM2 (Widada, et al., 1988), TPM3 (MacLeod, et al., 1986; Reinach & Macleod, 1986). TPM4 was sequenced from a 248 amino acid cDNA, cytoskeletal nonmuscle isoform (MacLeod, et al., 1987). Isoforms are different expressions of a tropomyosin gene. Each tropomyosin gene encodes different isoforms in the different types of muscle.

There are basically two types of muscle, striated and smooth muscle. Striated muscle consists of three major types, fast (psoas-glycolitic fast or gastrocenemius-oxidative

fast), slow (soleus) and cardiac muscle. There are two subtypes of cardiac muscle, atrial and ventricle muscle (Gunning, et al., 1990; Wolfe, 1993). This accounts for the six different possible isoforms produced by the tropomyosin genes ie. (1. fast, 2. slow, 3. atrial, 4. ventricle, 5. smooth, 6. non-muscle). The expression of TPM1, 2, and 3 is well defined in mammalian muscle fibroblasts. There is little data on the expression of TPM4 in muscle except for the detection of muscle-like exons in the human gene and the observation of a different sized transcript in total rat skeletal muscle. In contrast, non-muscle isoforms are poorly defined (see Table 1.1 for a summary of the TPM isoforms) (Gunning, et al., 1990; Libri, et al., 1990).

Eukaryotic genes consist of exons and introns, while prokaryotic genes have no introns. The exons are the regions of the gene that are transcribed to produce mature mRNA and usually contain coding information for protein translation. Introns are regions in between the exons that are not translated to produce a protein product. The gene is transcribed by the protein RNA polymerase into mRNA or the complementary sequence of the gene strand transcribed. The mRNA strand sequence is then spliced so the introns are removed and the mature mRNA is translated into a protein by ribosomes (Gelehrter & Collins, 1990; Micklos & Freyer, 1990; Wolfe, 1993).

The TPM isoforms in the different types of muscle and non muscle are produced by differential RNA splicing of the genes. This means the gene has the same number of exons or protein coding regions, but when the whole gene is transcribed, the mRNA is spliced alternatively into different messenger RNA (mRNA) fragments, which are then translated into the different protein isoforms. TPM3 consists of thirteen exons and the gene is around 42 kb long (see Figures 1.3 & 1.4) . Five exons are common to muscle and non-muscle cells, while three are unique to non-muscle cells (Clayton, et al., 1988; Gunning, et al., 1990; MacLeod & Gooding, 1988; MacLeod, et al., 1987). Alternative splicing occurs at three distinct points in the gene family, as seen on Figure 1.4. It is suggested the other TPMs have a similar gene structure with alternative splicing producing the different isoforms. In particular, TPM2's alternative splicing only occurs

on exons 6 and 9, while exon 2 does not undergo alternative splicing (Widada, et al., 1988). Two groups of tropomyosin isoforms have been described: there are 284 amino acids present in muscle and non-muscle isoforms and there are 248 amino acids that are unique to non-muscle cells (see Table 1) (Clayton, et al., 1988; Gunning, et al., 1990; MacLeod & Gooding, 1988; MacLeod, et al., 1987; Novy, et al., 1993; Ruiz-Opazo & Nadal-Ginard, 1987; Ruiz-Opazo, et al., 1985).

Table 1.1

Organisation Of The Human Tropomyosin Gene

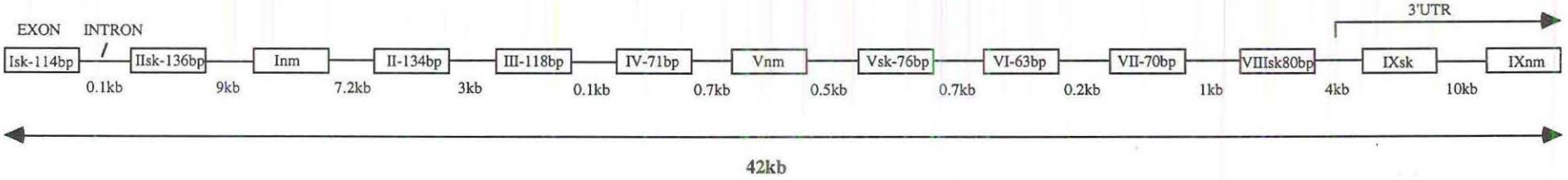
GENE	AMINO ACIDS	ISOFORMS	
TPM1	284	(i)	α-Tmf (fast twitch muscle)
Chrom. 15	284	(ii)	Tm2 (non-muscle)
(Eyre, et al., 1994)	284	(iii)	Tm3 (non-muscle)
TPM2	284	(iv)	β–Tm (striated muscle)
	284	(v)	Tm1 (non-muscle)
TPM3	284	(vi)	α-Tms (slow twitch muscle)
Chrom. 1	248	(vii)	Tm5 (non-muscle)
(Wilton, et al., 1995)			
TPM4*	248	(viii)	Tm4 (non-muscle)
19?	285?	(ix)	Has the potential to encode a muscle Tm
(Laing, 1994b)			

*(Tropomyosin 4 may be different in the number of exons and the splicing pattern)

(Gunning, et al., 1990; Libri, et al., 1990)

FIGURE 1.3

The Exons and Introns in TPM3.

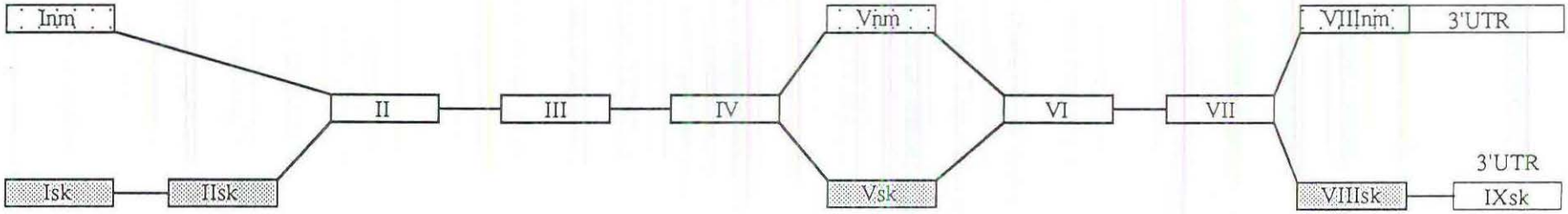


(After Clayton et al., 1988)

FIGURE 1.4

Alternative Splicing of the TPM3 Gene

The gene consists of 13 exons, only five of which (open) are common to both isoforms. The remaining exons are alternatively spliced to produce the skeletal muscle and non-muscle isoforms. Alternative splicing occurs at 3 distinct points in the gene and on a mutually exclusive basis. The 3' untranslated region is indicated as 3'UTR (After Clayton et al., 1988).


Inm
Isk
IIsk
II
III
IV
Vnm
Vsk
VI
VII
VIIInm
3'UTR
VIIIsk
3'UTR
IXsk

1.4 SIGNIFICANCE OF LOCALISING TPM2

There are many inherited muscular diseases which have been localised in the genome by linkage analysis, but for which no disease-causing mutation in a gene has been found. Each muscle-specific gene is possibly associated with an inherited muscle disease. Precise mapping and localisation of muscle genes in the human genome, therefore becomes important in relation to mapping inherited muscle diseases (Eyre, et al., 1993).

The alpha-tropomyosin gene TPM1 has recently been localised to 15q22 (Eyre, et al., 1994) and has been shown to be mutated in some cases of familial hypertrophic cardiomyopathy (Thierfelder, et al., 1994). The alpha-tropomyosin gene TPM3 has been recently localised to 1q22-q23 (Wilton, et al., 1995) and has been shown to be mutated in a family with autosomal dominant nemaline myopathy (Laing, et al., 1994).

Mapping muscle genes may also have implications for developing diagnostic tests for these diseases, especially prenatal tests and genetic counselling for people affected (Akkari, et al., 1994). By localising TPM2 it also lays a foundation for better understanding the role of tropomyosins in muscle and non-muscle tissues.

The context of this project in collaboration with the Australian Neuromuscular Research Institute (ANRI) was to localise TPM2, a possible candidate gene for the inherited muscle disease, autosomal dominant nemaline myopathy. While this project was being carried out, it was found that a point mutation in TPM3 caused one form of autosomal dominant nemaline myopathy in a Western Australian family (Laing, et al., 1994). Yet it is still important to map TPM2, as it may be a candidate gene for other muscle diseases, including other nemaline myopathies.

1.5 PROJECT OUTLINE

There were six major components of the project:

1. To produce a sequence tagged site (STS) that is unique to only the TPM2 gene.
2. To physically map the human skeletal TPM2 gene by initially using this STS in a somatic cell hybrid panel.
3. To refine the mapping by using the STS in FISH to human metaphase chromosome spreads, or if 3 does not work, to:
4. Use the STS as a probe to isolate pure genomic clones from a genomic library, for use in FISH of human metaphase chromosome spreads, to physically map the location of the TPM2 gene more precisely.
5. To identify a polymorphism in the TPM2 gene, so it could be used in linkage studies and entered on the CEPH map.

CHAPTER 2

DEVELOPMENT OF AN STS FOR HUMAN SKELETAL MUSCLE β-TROPOMYOSIN (TPM2)

2.1 INTRODUCTION

The aim of developing a specific STS for human skeletal β-tropomyosin (TPM2) was to use it on a somatic cell hybrid panel, for use in FISH directly, or as a probe to isolate a larger genomic clone from a library that contains all or part of the TPM2 gene, for use in FISH.

2.1.1 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a fairly recent development in molecular biology that amplifies a short sequence of DNA around a billion times between two primers (Mullis, et al., 1986). The standard PCR can amplify a product up to approximately 6 kb and from less than picograms (10^{-12}g) amounts of specific DNA, to around microgram (10^{-6}g) amounts, which can then be easily measured by agarose gel electrophoresis. The primers are chemically synthesised oligonucleotides, 20-35 nucleotides long with a free hydroxyl group present on the 3' end of the primer. The 3' end of a DNA strand, is where the ribose sugar terminates the alternate phosphate-sugar DNA backbone. The opposite 5' end of the strand terminates with a phosphate group, except that primers are synthesised with the 5' end dephosphorylated (Wilton, 1993).

If a primer is 20 nucleotides long with a random sequence, then because there are four nucleotides possible at each site, the chance that a primer will have the same sequence in two places in the genome is $(1/4)^{20}$ x $(1/4)^{20}$ or $1x10^{-26}$. Because there are $3x10^{9}$ bases in the genome, then a 20mer primer should usually have more than enough sequence to be unique in the genome, by a factor of 10^{17}. The specific amplified PCR product is

referred to as a STS as the primers and the sequence amplified between them is unique (Olson, et al., 1989).

The reaction takes place by heating the target DNA which denatures the strands. The reaction is then cooled and the primers which are present in high concentration anneal to their complementary sequence. A polymerase enzyme attaches to the 3' hydroxyl group of the primer and extends along the target sequence adding complementary nucleotides present in the reaction mixture to make the template double stranded. DNA synthesis proceeds in the 5' to 3' direction (see Figure 2.1). The polymerase does this about one thousand bases per minute. The particular polymerase enzyme, is derived from a bacteria (*Thermus aquaticus*) found in hot springs, so it can withstand high temperatures. The second primer is designed to anneal to the complementary strand of the first primer and its hydroxyl group is also on the 3' end of the primer. In this way the polymerase extends the template between the two primers. With alternate heating and cooling the template DNA is continually denatured and extended and therefore is multiplied in an exponential fashion, with the new product acting as a template for the next. The polymerase will extend past the other primer but in an arithmetic fashion, while the template between the primers will increase in an exponential fashion, so most of product DNA is of a defined length and can be measured by gel electrophoresis. After 30 cycles, taking about five minutes each, the target DNA between the primers is increased a billion fold in a small 10-100 µL eppendorf tube. The reaction efficiency is reduced after about 40 cycles, unless new enzyme and primers are added (Doggett, 1994; Gelehrter & Collins, 1990; Saiki, 1990; Wilton, 1993).

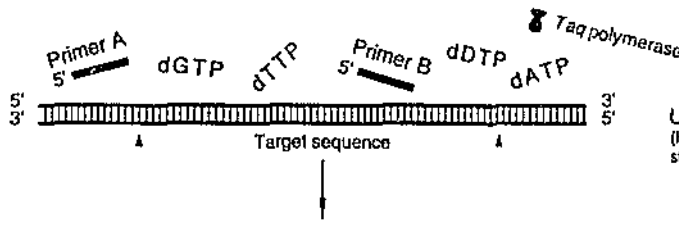
2.1.2 Primer Selection and Criteria

The exon sequence of TPM2 had already been determined (Widada, et al., 1988). Widada and his colleagues achieved this by obtaining transcribed mRNA of TPM2 in muscle tissue and using the enzyme reverse transcriptase to produce a complementary strand of DNA or cDNA copy of the TPM2 gene. They produced a complete cDNA

FIGURE 2.1

The Polymerase Chain Reaction (After Doggett, 1994).

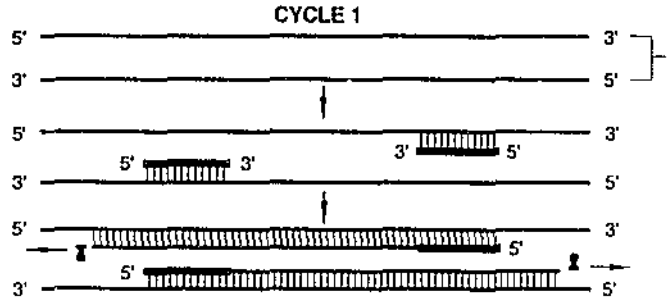
Reaction mixture includes DNA sample; two single-stranded primers, each with a 20-base sequence complementary to the 3' end of one strand of the target sequence; heat stable *Taq* polymerase; and deoxyribonucleoside triphosphates (dNTPs).



Unamplified DNA
(Hydrogen bonds between the two DNA strands are shown as vertical lines)

CYCLE 1

Phase 1
Denature unamplified DNA at 95°C to form single-stranded templates.



Template strands

Phase 2
Anneal primers to template at about 60°C.

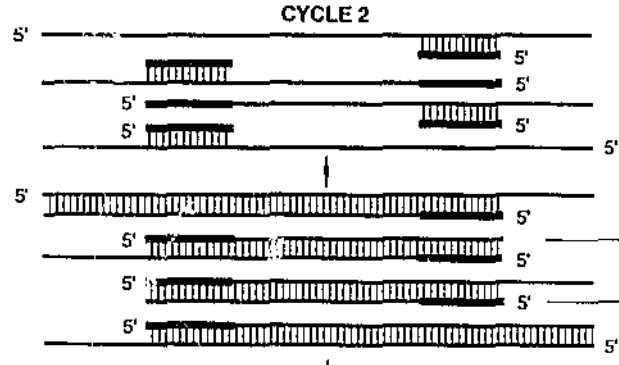
Each primer binds to its complementary sequence at the 3' end of a strand of the target sequence.

Phase 3
Synthesize new strands at 72°C.

Each new strand is synthesized in the 5'-to-3' direction.

CYCLE 2

Phases 1 and 2
Denature products of Cycle 1 and anneal primers to template strands.

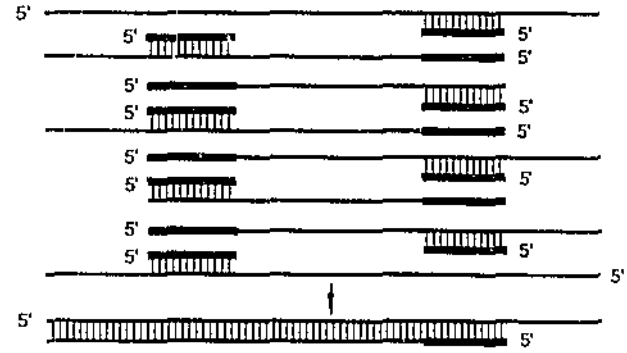


Phase 3
Synthesize new strands.

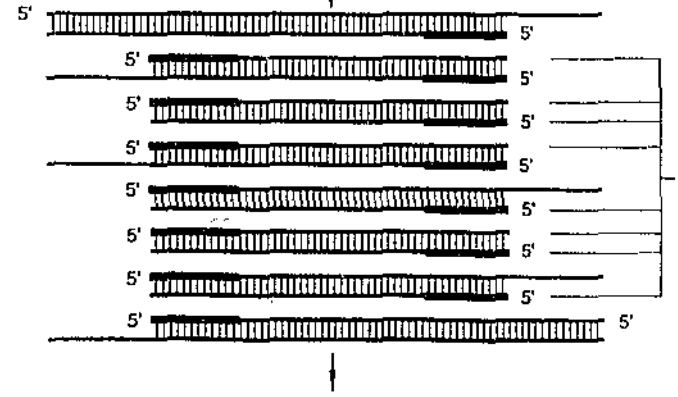
First two template strands that span the target sequence only

CYCLE 3

Phases 1 and 2
Denature products of Cycle 2 and anneal primers to template strands.



Phase 3
Synthesize new strands.



Eight template strands that span the target sequence only

Continue for 20 to 30 cycles to produce over 10^6 copies of target sequence.

sequence of the adult skeletal isoform of human skeletal muscle β-tropomyosin, or TPM2. Likewise, the exon sequence of TPM 1 (MacLeod & Gooding, 1988), TPM3 (Clayton, et al., 1988) and TPM4 (MacLeod, et al., 1987) have been determined. Using the TPM2 sequence, primers can be selected and synthesised for use in the polymerase chain reaction (PCR), to amplify a segment of the TPM2 gene.

Primers must be specific for amplifying a segment of the human skeletal muscle TPM2 gene, so no other non-muscle TPM2 isoforms or pseudogenes are amplified. Primers were synthesised that had the following criteria:

2.1.2.1 Primer sequence unique from other TPM genes

The primers must be designed to have a unique sequence that is different from the other three tropomyosin genes, so the other genes are not mistakenly amplified.

2.1.2.2 Primer sequence unique to muscle TPM2 isoform to avoid amplifying pseudogenes

A primer that includes specific skeletal muscle exon sequence that is spliced with the human skeletal TPM2 gene, will ensure that no nonmuscle TPM2 isoforms are amplified and that the STS is muscle specific (Clayton, et al., 1988; Widada, et al., 1988). This will also avoid amplifying pseudogenes.

Pseudogenes are DNA sequences that have the structure of expressed genes and were, presumably, once functional, but have acquired one or more mutations during evolution that render them incapable of producing a functional protein product. There are two major classes of pseudogenes (Singer & Berg, 1991).

The first class of pseudogenes can be silent, or they may be transcribed and even translated into aberrant polypeptides. They are often found closely linked to the

corresponding functional gene and may be flanked by sequences homologous to those that flank the corresponding functional gene. Such pseudogenes usually contain introns and appear to be the result of tandem duplications of DNA segments (Singer & Berg, 1991). An example of these types of pseudogenes are those found in the α and β-globin gene clusters (Gelehrter & Collins, 1990). Most gene families or clusters have members that are pseudogenes that usually represent a small minority of the total gene number (Lewin, 1994). Usually these types of pseudogenes do not affect the localisation of a gene, because they are so close to the real gene and FISH mapping is broad scale, localising a gene to a chromosomal band. On metaphase spreads, FISH resolution is 10-20 million bp, or to a chromosomal band, which is 0.3-0.6% of the genome, or approximately 8-16% of an average chromosome (Davies & Read, 1992). So if this type of pseudogene was mistakenly amplified and used as a probe for FISH, it would usually not affect the mapping location of the real gene.

There is another class of pseudogenes, called processed pseudogenes which have a similar sequence to the real gene, except only the exon sequence of the gene is conserved like a cDNA copy (ie. there are no introns and no protein product is produced). They are not generally linked to the corresponding functional gene but are dispersed throughout the genome, even to different chromosomes (Lewin, 1994; Singer & Berg, 1991). A theory for the presence of this type of pseudogene is that they were expressed mRNA that has been reverse transcribed back into DNA by the enzyme reverse transcriptase, which is present in retroviruses and reinserted in the genome. Since they may occur in other parts of the genome it is important that they are not mistakenly amplified or this will interfere with localising the TPM2 gene (Gelehrter & Collins, 1990; Singer & Berg, 1991). To detect this class of pseudogenes, primers can be used to amplify from genomic DNA, which includes the exons and introns and this can be compared using the same primers on cDNA, which only includes exon sequence. Any pseudogenes amplified would be smaller than the expected STS as they don't include the introns and their size can be predicted from the cDNA sequence. A pseudogene could then be detected by agarose

electrophoresis since their products would be smaller and migrate faster than the genomic STS (Eyre, et al., 1994; MacLeod & Gooding, 1988).

For processed pseudogenes to be inherited, they had to be mRNA that was reverse transcribed in the germ line cells, or in the cells that gave rise to germ cells. Usually, it is only transcribed general metabolic genes that produce this class of pseudogenes. Because muscle genes are not expressed in germ line cells, then there are likely to be no muscle-specific TPM2 exons present as pseudogenes. Therefore, primers synthesised from unique muscle specific exon sequence, should exclude amplifying processed pseudogenes. However, the non-muscle TPM2 isoforms are likely to produce pseudogenes as they are present in germ line cells. Clayton et al. (1988) found at least seven non-allelic TPM3 pseudogene sequences in the human genome, all derived from the nonmuscle isoform Tm5 (see Table 1). No such pseudogene sequences were found being derived from the TPM3 muscle specific exons. Therefore, by including a primer in a muscle-specific exon, specific to human skeletal muscle TPM2 and not the nonmuscle isoforms, excludes the chance of localising a pseudogene (Eyre, et al., 1994; Singer & Berg, 1991).

2.1.2.3 STS to include intronic sequence

If one primer is located in one exon and the other primer in another exon so intronic sequence is included, that will make the STS unique to TPM2. Intronic sequence is less conserved over evolutionary time than the exon sequence, as introns are not essential to protein synthesis and have little selection pressure at the phenotypic level (Lewis, 1994). This means the intron sequence in the STS will make it more unique than just having exon sequence alone. Ideally the primers should be designed to have intron sequence, but because the intron sequence was not known, only exon sequence could be used. By having intronic sequence in the STS, when it is used as a probe, excludes the possibility of isolating processed pseudogenes in a genomic library and localising processed pseudogenes for FISH (Eyre, et al., 1994).

2.1.2.4 Primer's GC ratio

The guanine and cytosine ratio of the primers (GC ratio) should ideally be 50:50 with the adenine and thymine ratio (AT ratio). This is because guanine and cytosine form three hydrogen bonds in double stranded DNA, while adenine and thymine form two hydrogen bonds (Freifelder, 1987). If the GC ratio is higher than the AT ratio then the primer will anneal at a higher temperature because there are more hydrogen bonds. If the AT ratio is higher then a lower annealing temperature is required, as there would be less hydrogen bonds. The GC ratio should be noted so the cycling conditions can be altered accordingly (Wilton, 1993).

2.1.2.5 Secondary and tertiary primer structures

The primers should be designed to avoid internal secondary and tertiary structures so they don't fold upon themselves and inhibit the PCR reaction (Wilton, 1993).

2.1.2.6 Primer complementarity

The primers should be designed to avoid complementarity between them otherwise they will bind to each other and not the target DNA, which is called a primer dimer (Wilton, 1993).

Once a human skeletal muscle specific TPM2 STS has been produced, it can be used on a somatic cell hybrid panel to localise the TPM2 gene to a chromosome, or part of a chromosome. An STS produced that is larger than 1 kb can be used directly in FISH for more precise mapping. If it is smaller than 1 kb, or it does not work in FISH, then the STS can be used to isolate a larger clone from a human genomic library (Akkari, et al., 1994).

2.2 MATERIALS AND METHODS

2.2.1 Primer Selection

The sequence of TPM2 is given in Appendix 1, from which the primers were selected and chemically synthesised, by ANRI.

Table 2.1

Primers Used For Amplifying TPM2

Sequence 5'- 3'	Primer No.	Exon
AACGCCATCGACCGCGCCGAG	DS 840.1skF	1 (Skeletal muscle-specific) Forward
AAGTATTCTGAATCCGTGAAG	DS 841.2skR	2 (" Muscle-specific) Reverse
TTGAAATCCCTGGAGGCCCAG	DS 752.5skF	5 (" Muscle-specific) Forward
ATTCCACCAAAGAAGATAAA	DS 869.6F	6 (Forward)
GCTGAGACCCGAGCAGAGTT	DS 868.7F	7 (Forward)
GAAAACCATCGATGACCTAGAA	DS 753.7R	7 (Reverse)
AAGTCTATGCCCAGAAGATG	DS 870.8skF	8 (" Muscle-specific) Forward
AGTTACTGTAGTGGAGGGAG	DS 866.8skR	8 (" Muscle-specific) Reverse
CCGTGACCGAAGTAGGAAAT	DS867. 3'UTR.R	3'UTR (Reverse)

A combination of these primers (see Table 2.1) were used in a PCR reaction on genomic DNA to see if a TPM2 STS was produced. The combination of primers was selected according to the criteria listed in the introduction (2.1.2). The expected STS size (see Table 2.2) was deduced from the structure of the TPM3 gene in Figure 1.3.

Table 2.2

Primer Combinations Used To Produce An STS

Primers			Expected STS Size (kb)
1skF	(DS 840) - 2skR	(DS 841)	~0.2
5skF	(DS 752) - 7R	(DS 753)	~1.0
5skF	(DS 752) - 8skR	(DS 866)	~2.0
5skF	(DS 752) - 3'UTR.R	(DS 867)	~2.2
6F	(DS 869) - 8skR	(DS 866)	~1.4
6F	(DS 869) - 3'UTR.R	(DS 867)	~1.5
7F	(DS 868) - 3'UTR.R	(DS 867)	~1.0
8skF	(DS 870) - 3'UTR.R	(DS 867)	259 bp

2.2.2 TPM2 PCR Conditions

For a 25 µL Reaction:-		**Final Conc.**
Target Genomic DNA (50 ng)	5.0 µL	2 ng/µL
5X 2^{200} Buffer (buffer, nucleotides and $MgCl_2$)	5.0 µL	2 mM, 200 µM
(5X *Tth* buffer, 10 mM $MgCl_2$, 1 mM dNTP's)		
Tth DNA polymerase (Biotech) (5.5 units/mL)	0.1µL	0.22 u/µL
Primer mix (10 ng/µL of each primer)	2.0 µL	0.8 ng/µL
H_2O to 25 µL	12.9 µL	
TOTAL	25.0 µL	

2.2.2.1 PCR cycling conditions: 2 step on automatic thermal cycler

Two thermal cyclers were used throughout the project for PCR reactions. The MJ Research Minicycler™ (Bresatec) required the addition of paraffin oil to the reactions to prevent evaporation during cycling. The other cycler was the MJ Research PTC-100 Programmable Thermal Controller (Bresatec), which had a hot bonnet, so the addition of paraffin to the reactions was not necessary.

		time	
temp	94°C	5 min	30 sec (denaturing)
	58°C		6 min (annealing and extension)
# cycles		x1	x35
	4°C		indefinitely

2.2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis separates a wide range of DNA fragments depending on the agarose concentrations.

0.7% agarose	10 to 0.8 kb	
1.0% agarose	6 to 0.4 kb	
2.0% agarose	4 to 0.2 kb	
3.0% agarose	2 to 0.1 kb	(Wilton, 1993)

Because the PCR STS was 1.5 kb, a 2% agarose gel was chosen. 4 µL of PCR product was run with 1 µL 5X agarose loading buffer on 2% agarose gel, in 1X TAE buffer, at 100V, for 45 min (see Appendix 2 for buffers used). The Biorad 3000Xi power pack was most often used, with Biorad agarose gel buffer tanks, gel trays and combs, though other power packs and gel trays were used.

2.2.4 Staining Agarose Gels

The gel was then stained in 0.5 μg/mL of ethidium bromide for 10 min to observe the DNA bands on a ultraviolet (UV) light, 305 nm transilluminator (Fotodyne Inc.). The ethidium bromide interchelates between the G:C bases of DNA and becomes associated with the DNA bands in the gel. The DNA absorbs the UV light and transfers this energy to the ethidium bromide and emits light in the red-orange region of the visible spectrum (Wilton, 1993). The gel was photographed using a mounted Polaroid CU-5 Land Camera (focal length 127 mm) at maximum aperture (4.7) for 0.25 sec. The black and white film used was Polaroid Type 667.

2.2.5 DNA markers

To determine the size of the DNA fragments in agarose gel electrophoresis, DNA markers of known sizes are run with the PCR products. Four markers were used in this project for agarose gel electrophoresis. Lambda (λ) bacteriophage when cut with the restriction enzyme *Hind* III provided a high molecular weight marker and when cut with *Pst* I provided high to medium weight markers. The plasmid *pUC* 19 cut with the restriction enzyme *Hpa* II and pGEM® (Promega) produce low molecular weight markers.

2.2.5.1 Lambda bacteriophage cut with Hind III

For a 50 μL Reaction

10X Buffer B (Promega)	5 μL
Acetylated BSA (1 mg/mL)	5 μL
Spermidine (0.1 M)	0.8 μL
λ Phage (400 ng/μL) (Promega)	38.2 μL
Hind III (12 u/μL)	1 μL

2.2.5.2 Lambda bacteriophage cut with *Pst* I

For a 50 µL Reaction

10X Buffer H (Promega)	5 µL
Acetylated BSA (1 mg/mL)	5 µL
Spermidine (0.1M)	0.8 µL
λ Phage (400 ng/µL) (Promega)	38.2 µL
Pst I (12 u/µL)	1 µL

2.2.5.3 *pUC* 19 cut with *Hpa* II

For a 50 µL Reaction

10X Buffer A (Promega)	5 µL
Acetylated BSA (1mg/mL)	5 µL
Spermidine (0.1M)	0.8 µL
pUC 19 (500 ng/µL) (Promega)	38.2 µL
Hpa II (12 u/µL)	1 µL

(Promega. 1991)

Note, enzymes have specific pH, temperature and ionic requirements, hence the 10X buffer which contains Na^+ and Mg^{2+} ions, Tris-HCl as a pH buffer and diothiothreitol. The acetylated bovine serum albumim (BSA) is a source of neutral protein to add to enzyme reactions. Enzymes in a reaction containing less than 0.1 mg/mL protein, rapidly denature in dilute reactions. BSA should be used at a final concentration of 0.1 mg/mL (Sambrook, et al., 1989).

FIGURE 2.2

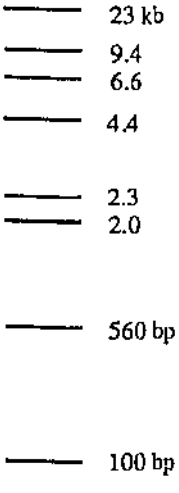
DNA Molecular Weight Markers Used

DNA Source	Restriction Enzymes
(A) λ DNA	*Pst* I
(B) λ DNA	*Hind* III
(C) pUC 19	*Hpa* II
(D) pGEM[1]	*Hinf* I, *Rsa* I, *Sin* I

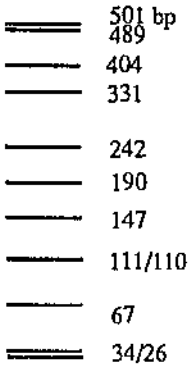
[1](Promega, 1993/94)

(A) Lambda cut with Pst I

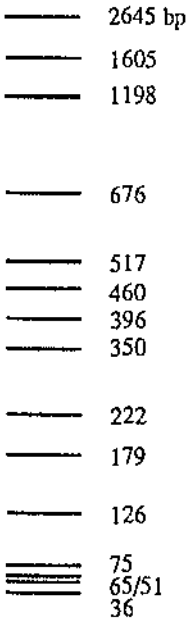
(B) Lambda cut with Hind III



(C) pUC 19 cut with Hpa II



(D) pGEM



The restriction digest reactants were mixed lightly in a 0.6 mL eppendorf and incubated at 37°C for 1 hour to allow the enzyme digestions to take place. One unit of restriction enzyme activity is defined as the amount of enzyme required to produce a complete digest of one microgram of substrate DNA (or fragments) in 60 minutes at the appropriate assay temperature in a 50 µL reaction volume (Promega, 1993/94). Therefore, 1 µL of each restriction enzyme was more than enough to ensure complete digestion. The tubes were then heated to 95°C for 5 min to denature and deactivate the enzymes. Each restriction digest was then added to 50 µL of Ficoll 1X loading buffer. Usually 3 µL of a marker was loaded in a well for agarose electrophoresis, or was determined empirically. Note that pGEM marker was supplied by Promega.

2.2.6 Purifying the TPM2 PCR STS DNA

Once a TPM2 PCR STS had been developed, it had to be confirmed that the STS was from the TPM2 gene. The most precise method for doing this was to sequence the STS. Before this could be done, the PCR product had to be purified for sequencing. This involved running the PCR product on a low melting point agarose gel (LMPAG) (Biorad) and cutting the DNA band out. The DNA in the agarose was then purified in a resin column (Wizard™ PCR Preps Purification System - Promega). The purified DNA was re-run on a gel and the absorbance of the purified DNA was checked to measure the DNA concentration, before the template was used for sequencing.

2.2.6.1 Low melting point agarose gel (LMPAG)

The TPM2 PCR STS is firstly amplified in a 50 µL reaction to produce sufficient template.

For a 50 µL Reaction:-		Final Conc.
Target Genomic DNA (50 ng)	5.0 µL	1 ng/µL
5X 2^{200} Buffer (buffer, nucleotides and $MgCl_2$)	10.0 µL	2 mM, 200 µM
(5X Tth buffer, 10 mM $MgCl_2$, 1 mM dNTP's)		
Tth DNA polymerase (Biotech) (5.5 units/mL)	0.2 µL	0.22 u/µL
Primer mix (10 ng/µL of each primer)	4.0 µL	0.8 ng/µL
H_2O to 25 µL	17.9 µL	
TOTAL	50.0 µL	

The cycling conditions were the same as those used in 2.2.2.1.

The amplification was run first on a normal 2% agarose gel to see if it had worked, as LMPAG is very expensive. 3 µL of PCR product was run with 0.6 µL 5X Ficoll agarose loading buffer on a 2% agarose gel, in 1X TAE buffer, at 100V, for 45 min. The gel was stained in ethidium bromide for 10 min and photographed. 5 µL of 5X Ficoll loading buffer was added to the rest of the reaction (47 µL) and run on a thickly poured (~1 cm) 2% LMPAG (Bio-Rad), as for a normal 2% agarose gel (2.2.3).

The LMPAG was then stained with 0.5 µg/mL ethidium bromide for 10 min and the DNA bands were observed on the UV transilluminator. The bands were cut out using a clean sterile scalpel blade as quickly as possible, because the UV light can nick the DNA strands. The band was isolated in about 300 µL of agarose.

2.2.6.2 Purification of DNA using Wizard™ PCR Preps DNA Purification System (Promega)

The Wizard™ PCR Preps System (Promega) purifies double-stranded DNA from contaminants such as primer-dimers and amplification primers, unincorporated nucleotides and enzymes (Promega, 1993).

For each PCR product:-

1. The DNA band was excised quickly (to avoid nicking the DNA) from the gel and placed into a 1.5 mL centrifuge tube.

2. The tube was heated at 70°C until the agarose completely melted.

3. 1 mL of Magic PCR Preps Resin was added to the melted agarose and vortexed for 20 sec to mix.

4. The minicolumn was placed on the end of a 3 mL syringe barrel, by the luer-lock extension of the minicolumn.

5. The plunger was removed and the resin/DNA mix was pipetted into the syringe barrel.

6. The syringe plunger was inserted slowly and the slurry was pushed through the minicolumn and into a 1.5 mL centrifuge tube.

7. The syringe was removed from the minicolumn and then the plunger was removed from the syringe.

8. The syringe barrel was reattached to the minicolumn and 2 mL of 80% isopropyl alcohol was pipetted into the syringe barrel to wash the column. The plunger was inserted and the isopropyl alcohol was pushed through the minicolumn.

9. The minicolumn was removed and placed on a 1.5 mL centrifuge tube and centrifuged at 12 000 xg, to dry the resin.

10. The minicolumn was transferred to a new 1.5 mL tube and 50 µL of H_2O was added to the minicolumn for approximately 1 minute (the DNA would remain intact on the minicolumn for up to 30 minutes).

11. The minicolumn and tube were centrifuged for 20 seconds at 12 000 xg, to elute the DNA into the tube.

12. The minicolumns and all old 1.5 mL tubes were discarded.

13. The sample was kept at 4°C or -20°C.

14. 2 µL of the Wizard prep sample was run on a 2% agarose gel with a marker to see the recovery of the product.

(Promega, 1993)

2.2.7 Measuring the absorbance of nucleic acids on a spectrophotometer

The absorbance of the purified DNA was measured on a Beckman DU 650 spectrophotometer and the concentration of DNA was calculated.

An absorbance of 1 at 260 nm $1A_{260}$ for double stranded DNA (dsDNA) is 50 ng/µL

" " $1A_{260}$ for single stranded DNA (ssDNA) is 33 ng/µL

" " $1A_{260}$ for RNA is 40 ng/µL

(Promega, 1991; Wilton, 1993)

Therefore, dsDNA concentration = absorbance x dilution factor x 50 ng/μL

and ssDNA " = " x " " x 33 ng/μL

Note, that any contaminating RNA at $1A_{260}$ will be read at 40 ng/μL and contaminating primers being ssDNA will be read at 35 ng/μL. So it was important to run the purified DNA on a gel to see if there was any contaminating RNA at the bottom of the lane in the gel.

The purity of the DNA sample can be measured for contaminants such as protein or phenol by the A_{260}/A_{280} ratio. Pure DNA will have an A_{260}/A_{280} ratio of 1.8-2.0. Phenol absorbs strongly at 270 nm and proteins absorb at 280 nm (Wilton, 1993). The absorbance curve can be observed on the spectrophotometer for peaks at 270 nm and 280 nm suggesting phenol and protein contamination respectively (Wilton, 1993).

2.2.8 Dideoxy Chain Termination Automatic Cycle Sequencing

2.2.8.1 Introduction

Automatic cycle sequencing is based on the Sanger dideoxy sequencing method (Sanger, 1981). The PRISM™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) was used. The kit produces chain terminated fragments from unlabelled primers extended by the thermostable *Taq* DNA polymerase. Each dideoxynucleotide (ddNTP) is labelled with a different fluorescent dye; ddATP-green, ddCTP-blue, ddGTP-yellow and ddTTP-red. These four ddNTPs are present in the reaction at approximately 1-2% of the concentration of the normal deoxynucleotides (dNTP's). The *Taq* polymerase extends the DNA from a single primer by incorporating the dNTP's until a fluorescently labelled ddNTP is incorporated in the chain causing the chain to terminate (Applied-Biosystems, 1993). This occurs at random, so from the primer, different chain lengths will be produced each with different fluorescently labelled ends (see Figure 2.3). A dideoxynucleotide has a hydrogen instead of an -OH group

linked to the 3'-carbon in the ribose sugar. Therefore there is an oxygen missing from the 3'-carbon. Dideoxynucleotides can be inserted during DNA replication, but synthesis stops at an insertion point because there is no 3'-OH for the addition of the next nucleotide in the chain (Wolfe, 1993). The four base reactions occur simultaneously in the one tube. Alternate heating and cooling in a thermal cycler causes the DNA to denature and become single stranded when heated. Cooling causes the primers to anneal to the template DNA and the polymerase extends the complementary DNA strand with dNTP's until a fluorescently labelled ddNTP is incorporated and causes the chain to stop. Heating again releases the primers, denatures the DNA and the cycle continues (see Figure 2.3). Because only a single primer is used per reaction, the DNA templates are linearly amplified. This is unlike the normal PCR that exponentially amplifies the target DNA. The accuracy of the sequencing can be compared by using the complementary primer to sequence in the reverse direction, as long as the other primer is within approximately 500 bases (Applied-Biosystems, 1993; Brown, 1994; Manomi, et al., 1992).

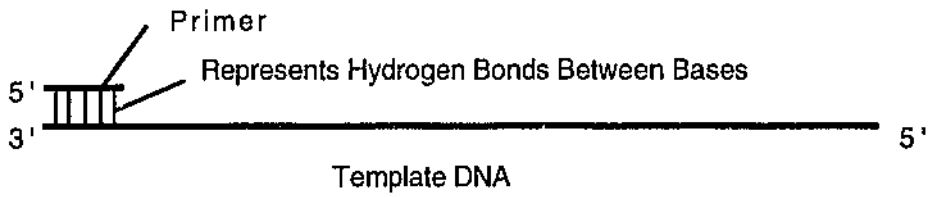
The unincorporated dye terminators are removed by organic extraction. The reaction products were loaded into a single lane and separated by acrylamide gel electrophoresis on the automatic sequencer. The smallest DNA end labelled chains travel the fastest through the gel, while the larger fragments take longer. As the fragments separate by size they pass through a fixed position in the gel. A laser excites the fluorescent dye of each coloured end-labelled DNA fragment as it scans back and forth across this area of the gel. A photomultiplier tube (PMT) detects the fluorescent light colours and converts it into an electrical signal that is transmitted to a computer for storage and processing. Each scan consists of four passes, once through each of the four coloured filters, which measures the colour of each end-labelled base as it passes through the gel. The raw data is analysed by the computer, which then prints the analysed sequence of the DNA template (Applied-Biosystems, 1993; Brown, 1994; Manomi, et al., 1992).

FIGURE 2.3

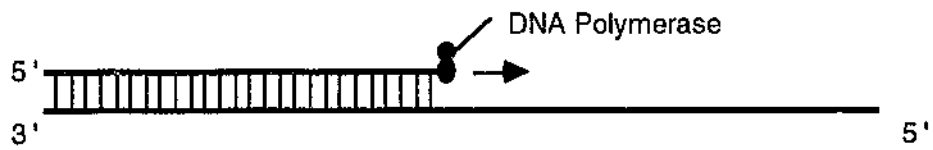
Dideoxy Chain Termination in Sequencing

(After Brown, 1994)

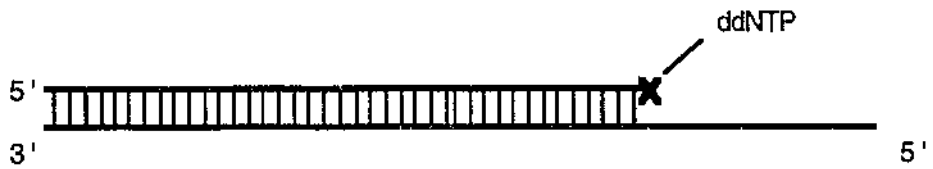
(A) Anneal the primer to the template



(B) Strand Synthesis



(C) Strand Termination



2.2.8.2 Cycle sequencing - dyedeoxy terminator cycle

For a 15 µL Reaction:-

	µL
"Prism Cocktail"	7.5
Primer (20 ng/µL)	2
Template (50-300 ng)	
H_2O up to 15 µL	
Paraffin overlay	1 drop

"Prism Cocktail" contains:-	Dye
ddATP -	Green
ddCTP -	Blue
ddGTP -	Yellow
ddTTP -	Red
Taq polymerase enzyme	
dNTP's and buffer	

Cycling Conditions

			time	
temp	94°C	15 sec	30 sec	denaturing
	50°C		1 min	(annealing)
	60°C		4 min	(extension)
# cycles		x1	x35	
	4°C		indefinitely	

2.2.8.3 Applied Biosystems Inc. sequencing clean-up to remove unincorporated ddNTPs

Upon completion of the cycling:-

Transferred sequencing reaction to a fresh tube (0.6 mL eppendorf) with minimal oil carryover

Added 80 µL water

Added 100 µL ABI phenol

Mixed thoroughly and centrifuged at 12 000 rpm for 5 min at 4°C

Transferred aqueous phase (top phase) to fresh tube

Repeated phenol extraction

Transferred aqueous phase to a fresh tube

Added 15 µL 2M Na Acetate pH 5.0
300 µL absolute ethanol

Mixed well and spun 12 000 rpm, 15 min, 4°C
(pre-chilling is not necessary)

Discarded supernatant (above pellet)

Washed pellet (small, clear and not coloured) with 70% Ethanol and used a pipet to remove as much ethanol as possible

Centrifuged again 12 000 rpm, 5 min, 4°C

Brief vacuum dried for 5-10 min (Speedvac Model SVC 100 - Savant)

Eppendorfs were capped and reactions can be left at this stage ready for sequencing loading

(ANRI, 1994)

The 1.5 kb product was sequenced using an ABI 373A DNA sequencer to confirm that the STS product was part of the TPM2 gene.

2.2.9 Using the Radioactive Random Labelled TPM2 STS on a Genomic Hybridisation Membrane

2.2.9.1 Random primer labelling introduction

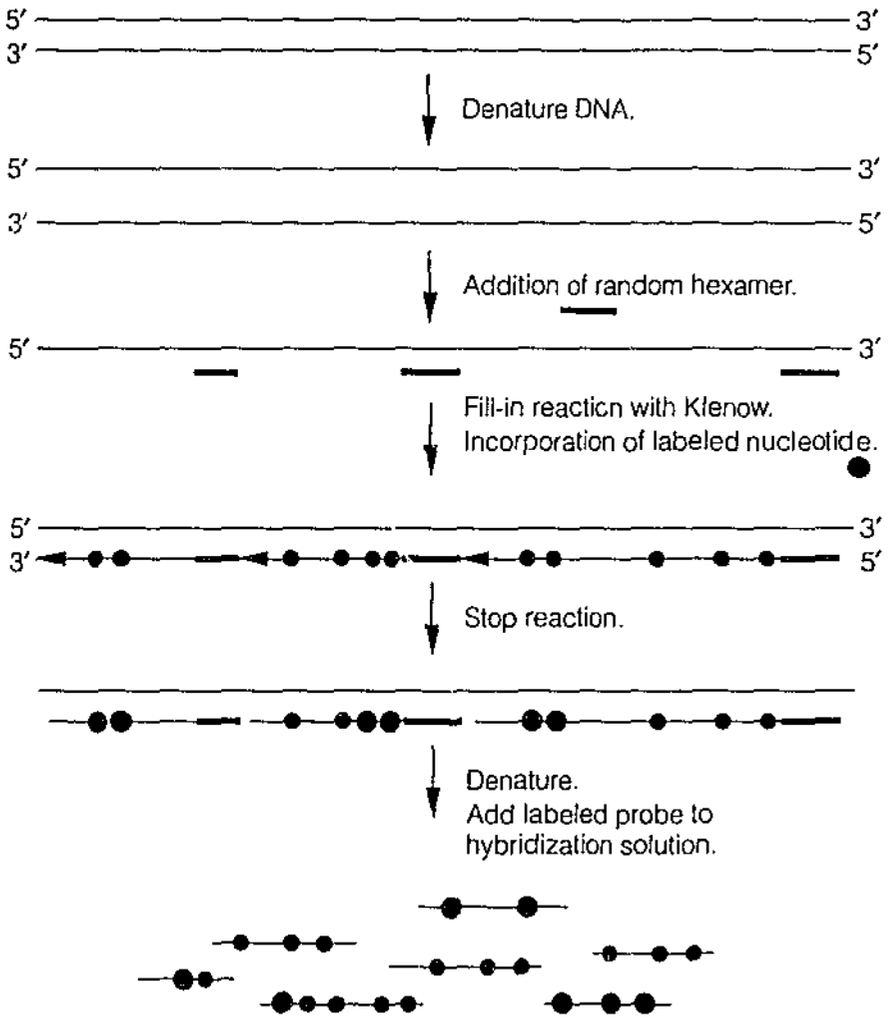
The TPM2 STS was radioactively labelled and hybridised with a genomic blot to see if the STS would hybridise to genomic DNA before it was used for FISH.

This Prime-a-Gene labelling system was developed by Feinberg and Volgelstein (1984), and is where a mixture of random hexanucleotides primers is used to prime DNA synthesis *in vitro* from any linear double stranded DNA template. The hexamer primers anneal to the DNA template at random positions and Klenow DNA polymerase extends from the random primers. A radioactive nucleotide such as (α-^{32}P) dCTP and normal nucleotides (dATP, dGTP, dTTP) are incorporated in the synthesis of a complementary strand which is labelled radioactively as the (α-^{32}P) dCTP is incorporated. Probes can be generated up to 50% of the length of the original template with a high specific activity (often > 10^9 cpm/μg) (see Figure 2.4) (Feinberg & Vogelstein, 1984; Promega, 1991).

FIGURE 2.4

Random Primer Labelling

(After Promega, 1993)


5′
3′
3′
5′
Denature DNA.
5′
3′
3′
5′
Addition of random hexamer.
5′
3′
Fill-in reaction with Klenow.
Incorporation of labeled nucleotide.
5′
3′
3′
5′
Stop reaction.
Denature.
Add labeled probe to
hybridization solution.

The STS is then denatured and the radioactively labelled complementary strand hybridises to a human genomic blot. The actual genomic blots used were prepared and supplied by ANRI, however the process of how they were made will be discussed.

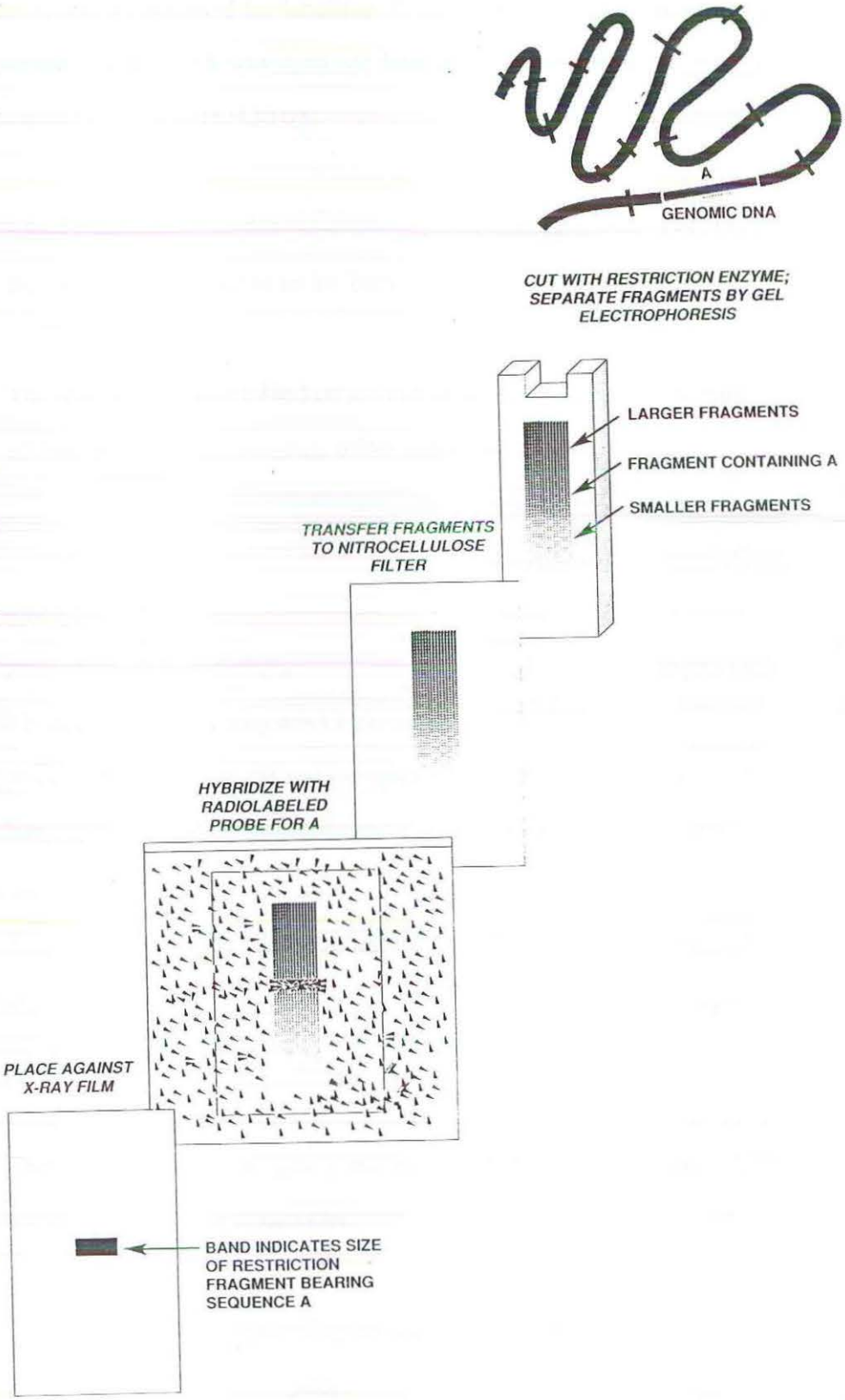
A genomic blot is a Southern Blot of total genomic human DNA. Isolated DNA from different people is digested with a restriction enzyme that cuts the DNA at a specific sequence producing different sized fragments. The digested DNA from different individuals is electrophoresed on an agarose gel in different lanes so that the DNA fragments separates according to size, the smallest fragments travelling the furthest and fastest. The gel is then rinsed in an acid solution that depurinates or 'acid nicks' the DNA, which cuts the DNA into smaller fragments at some of the purine bases (ie. adenine and guanine) so the DNA can travel through the gel more easily. The gel is then rinsed in an alkaline solution which denatures the DNA in the gel. The gel is then vacuum blotted so the depurinated and denatured DNA travels on to a nitrocellulose or nylon membrane where it hybridises to the membrane in the band pattern that was present in the gel. The membrane is then rinsed in a pH neutralising solution and dried (Gelehrter & Collins, 1990; Wilton, 1993).

The radioactive STS probe is denatured by heating and hybridised to the human genomic membrane where it anneals to the complementary sequence in the genome. The membrane is then washed to remove the excess or unincorporated probe on the membrane and then dried and autoradiographed. The probe may anneal at different bands on the membrane because the restriction enzyme may have cut the complementary DNA sequence at two or more places where the probe would anneal, so there may be several bands present on the autoradiograph (Gelehrter & Collins, 1990; Wilton, 1993) (see Figure 2.5).

FIGURE 2.5

Southern Blotting to Produce Genomic Blots

(After Gelehrter, 1990)


A
GENOMIC DNA
CUT WITH RESTRICTION ENZYME; SEPARATE FRAGMENTS BY GEL ELECTROPHORESIS
LARGER FRAGMENTS
FRAGMENT CONTAINING A
SMALLER FRAGMENTS
TRANSFER FRAGMENTS TO NITROCELLULOSE FILTER
HYBRIDIZE WITH RADIOLABELED PROBE FOR A
PLACE AGAINST X-RAY FILM
BAND INDICATES SIZE OF RESTRICTION FRAGMENT BEARING SEQUENCE A

2.2.9.2 Random primer labelling protocol

The following reaction is optimised for labelling 25 ng of DNA template, however when 25 ng of the purified TPM2 STS was used the labelling was too weak, so 5 µL of 24 ng/µL DNA template was used (ie. 120 ng).

1. The TPM2 DNA template was denatured by heating at 95-100°C for 2 min, then snap chilled in an ice bath.

2. The reaction was assembled on ice in a separate microcentrifuge tube by adding the following reagents in the order listed.

Component	Volume	Final Conc.
(a) Labelling 5X buffer	10 µL	1X
(b) Mixture of unlabelled dNTP's (dGTP, dATP, dTTP, 1 µL each (3 µL total)	2 µL	20 µM each
(c) Denatured DNA template (25 ng) but used	120 ng	2.4 ng/µL
(d) (α-^{32}P) dCTP (Bresatec) behind perspex screen (50 µCi, 3000 Ci/mmole)	5 µL	50 µCi
(d) Acetylated (nuclease free) BSA (10 mg/mL)	2 µL	0.4 µg/µL
(e) Klenow enzyme	5 units	0.1 u/µL
(f) Sterile H_2O to final volume 50 µL		

3. The reaction was mixed gently and incubated at room temperature (25°C) for 60 min in a PCR minicycler.

4. The reaction was terminated by heating to 95-100°C for 2 min and chilling in an ice bath. EDTA was added to 20 mM and used directly in the hybridisation reaction or stored at -20°C.

(Promega, 1991)

2.2.9.3 PEI strip test

The percentage incorporation of ^{32}P into the probe by random priming was checked by using PEI Cellulose thin layer chromatography (see Figure 2.6).

1. An approximate 8 cm strip of PEI paper was cut and a line was drawn in pencil 1 cm from the bottom of the strip and an "x" was marked in the middle of the line.

2. 0.5 µL of the random priming reaction was placed on the "x".

3. Approximately 5 mL of 0.75 M $K_2H_2PO_4$ (pH 3.0) was pipetted into a Coplin jar and the labelled PEI strip was placed in the jar so that only the top of the strip touched the side of the jar .

4. The $K_2H_2PO_4$ solvent front was allowed to move to the top of the strip.

5. The cpm of the strip were measured at the "x" and up the strip where the solvent had moved the unincorporated ^{32}P in dCTP in a tear fashion.

Efficiency labelled = cpm at "x" / (cpm up the strip + cpm at "x")

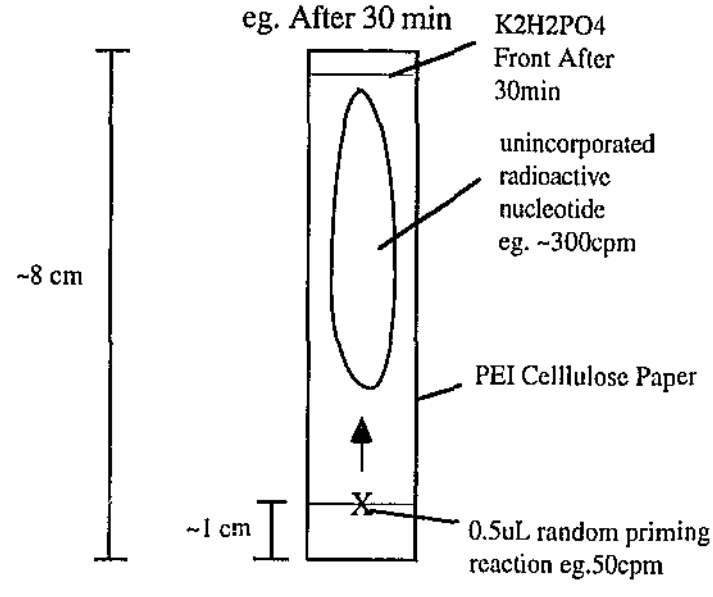
If the labelling efficiency was too low (0-20%) then the random priming reaction was repeated using more template (ie. >25 ng DNA).

2.2.9.4 Sephadex G-50 Spin Column

The purpose of the Sephadex G-50 spin column is to remove the unincorporated (α-^{32}P) dCTP in the random priming reaction. This step is not necessary unless the incorporation levels are low in the random priming reaction. The unincorporated (α-^{32}P) dCTP can be removed by size exclusion chromatography on Sephadex G-50 beads. The spin column

FIGURE 2.6

PEI Strip Test to Measure the % Incorporation of the Radioactive Nucleotide in the Probe


eg. After 30 min
K2H2PO4
Front After
30min
unincorporated
radioactive
nucleotide
eg. ~300cpm
~8 cm
PEI Cellulose Paper
X
~1 cm
0.5uL random priming
reaction eg.50cpm

leaves the probe free of unincorporated (α-^{32}P) dCTP and also reduces the content of DNA oligomers less than 70 bases in length. This therefore, reduces the background radiation when using hybridisation probes (Promega, 1991).

Protocols

1. A spin column was prepared by plugging a 3 mL syringe with a small piece of sterile non-absorbent cotton wool.

2. The 3 mL syringe was filled with Sephadex G50 and compacted with the plunger until it measured about 2 mL and the plunger was then removed.

3. 1 mL of STE (DNA buffer - see Appendix 2) was added to the top of the syringe and the syringe was placed in a 15 mL polypropylene conical tube and centrifuged at 2000 rpm for 5 min on a Beckman model TJ-6 centrifuge.

4. The syringe was placed in a new 15 mL tube and the random priming reaction (stopped) was added to the syringe and centrifuged at 2000 rpm for 5 min on a IEC model HN-5 centrifuge.

5. The syringe containing the unincorporated (α-^{32}P) dCTP was discarded which also contained DNA oligomers less than 70 bases in length.

6. The purified probe was pipetted from the 15 mL tube into a 0.6 mL eppendorf tube and denatured by heating to 94^{o}C for 5 min and then snap chilled on ice for 1 min.

7. The denatured probe was added to the prehybridisation solution and genomic blot.

(ANRI, 1994)

2.2.9.5 Prehybridisation of the genomic blot

The human genomic blots are prehybridised in a solution that is used to block non-specific binding of the probe to the membrane. The prehybridisation solution contains salmon sperm DNA that binds to the membrane everywhere else the immobilised genomic DNA did not bind, therefore blocking any unincorporated probe from non-specifically hybridising to the membrane (Wilton, 1993).

Prehybridisation solution protocol

10-20 mL of prehybridisation solution was used per membrane. The reagents were added in the following order:

Hybridisation Solution	for 1 membrane	final concentration
1. 5 M NaCl	4 mL	1 M
2. De-ionised formamide	10 mL	1 M
3. 1 M Tris pH 7.5	1 mL	50 mM
4. 10% SDS	2 mL	1%
5. 10 mg/mL Salmon Sperm DNA	0.2 mL	0.1 mg/mL [1]
6. 50% dextran sulphate	4 mL	10%[2]
Store at -20°C in aliquots		

[1] The salmon sperm DNA was boiled at 100°C for 5 min, and then snapped chilled on ice before adding to the solution. Salmon sperm DNA should have been cut to an average size of 500-700 bp.

[2] The formamide was freshly deionised by mixing for one hour at room temperature, with two spoonfuls of Mixed Bead Resin (Bio-Rad), then filtered through 305 mm diameter Postlip Paper (Hollingsworth & Vose Co. Ltd).

Formamide acts to keep the DNA probe single stranded (Wilton, 1993). The 10% dextran sulphate increases the rate of hybridisation approximately ten-fold, because the nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased (Sambrook, et al., 1989).

The 1 M NaCl maximises the rate of annealing of the probe with its target as hybridisation is increased in solutions of high ionic strength (Sambrook, et al., 1989).

The 1% SDS solution is a detergent and acts as a surfactant so the probe will anneal to the membrane target more easily (Sambrook, et al., 1989).

The 15 x 20 cm genomic blot was placed in a plastic envelop and 20 mL of prehybridisation solution was added to the bag and mixed around. The bag was heat sealed and air bubbles in the bag were pushed to one corner and heat sealed in. The bag with the prehybridisation solution and the genomic blot were placed in a 42°C shaking water bath (Grant model SU 5/6) for 1 hour. For newly made genomic blots or immobilised DNA on nylon membranes, the first time they are used for hybridisation they had to be prehybridised for approximately 4 hours (ANRI, 1994).

2.2.9.6 Hybridisation of the radiolabelled TPM2 STS probe to the genomic blot

The 50 µL of radiolabelled TPM2 STS probe (2.2.9.2) was then added to the prehybridisation solution after cutting one corner of the bag with scissors. The air bubbles were again removed by heat sealing in one corner and the probe was mixed around the bag with a glass rod. The air bubbles were removed to ensure every part of the membrane would come in contact with the probe. To avoid contaminating the water bath, the hybridisation bag was placed in a large tupperware container filled with water and placed in the water bath at 42°C and left shaking for 16 hours (ANRI, 1994).

2.2.9.7 Washing the membrane at increasing stringencies

The membrane was then washed at 42°C at increasing stringencies to remove the non-specific binding of the probe to the membrane. After each wash the membranes radioactivity was measured using a geiger counter (Mini Instruments Series 400 - mini-monitor GM tube). The membranes were washed until the counts per minute (cpm) fell to approximately 10-20 cpm.

1. The membrane was first washed in 200 mL of 2X SSC, the least stringent wash for 15 min at 42°C shaking in the water bath.

2. The second wash was in 2X SSC and 1% SDS for 15 min at 42°C shaking in the water bath.

3. The third wash was with 0.1X SSC and 0.1% SDS, the most stringent wash, for 15 min at 42°C. If the cpm's were already around 20 cpm then the third wash was not necessary. (ANRI, 1994).

Note that increasing the temperature of the wash also increases the washing stringency. The membrane was then blot dried with Whatman 3MM paper and wrapped in Glad Wrap. Autoradiography was performed with Dupont Cronex 4 X-ray film with Quanta III autoradiography intensifying screens at -80°C for an empirically determined time (Akkari, et al., 1994).

2.2.9.8 Stripping the genomic blots

The genomic blots after autoradiographing were stripped to remove the probe and all unspecific labelled DNA, so they could be used again.

1. The membrane was gently agitated in 500 mL of 0.4 M NaOH for 30 min at 42°C.

2. The membrane was transferred to a clean dish and gently agitated in 500 mL of 0.1X SSC, 0.1% SDS and 0.2 M Tris (pH 7.5) for 30 min at 42°C.

3. The membrane was briefly rinsed in 0.1X SSC and 0.1% SDS.

4. The activity of the membrane was checked and if at background, it was blotted dry and stored at 4°C.

2.3 RESULTS

The only primer combination in Table 2.1 that produced an STS from genomic DNA initially were primers 869 (exon 6) and 866 (exon 8sk) which amplified a 1.4 - 1.6 kb (1.5 kb) product (see Figure 2.7A). A blank was run where all the reactants were added except the genomic DNA.

This STS was then amplified in a 50 µL reaction and run on a 2% LMPAG (see Figure 2.7B). The DNA band was cut out and purified using Wizard™ PCR Preps DNA Purification System (Promega). 2 µL of the purified DNA was run on a 2% agarose gel, 100V for 30 min to determine how much product there was after purification (see Figure 2.7C) and the absorbance of the purified DNA was measured on a spectrophotometer (Beckman DU 650) to calculate the DNA concentration (see Table 2.3). The measured

FIGURE 2.7

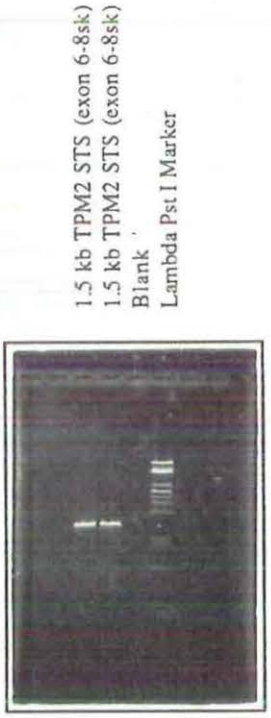
~1.5 kb TPM2 STS From exon 6 to 8sk (skeletal muscle specific exon)

(A) The STS Amplified from Two Genomic DNA Samples and Run on a 2% Agarose Gel at 100V, for 1 hr.

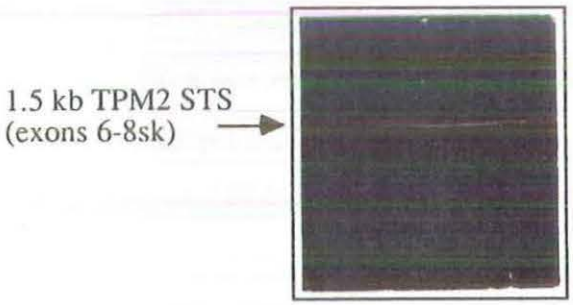
(B) The Pooled STS Samples Run on a 2% Low Melting Point Agarose Gel (LMPAG) at 100V, for 1 hr.

(C) The STS cut out from the LMPAG and Purified using Promega Wizard™ PCR Purification Preps System. 2% Agarose Gel, at 100V for 30 min.

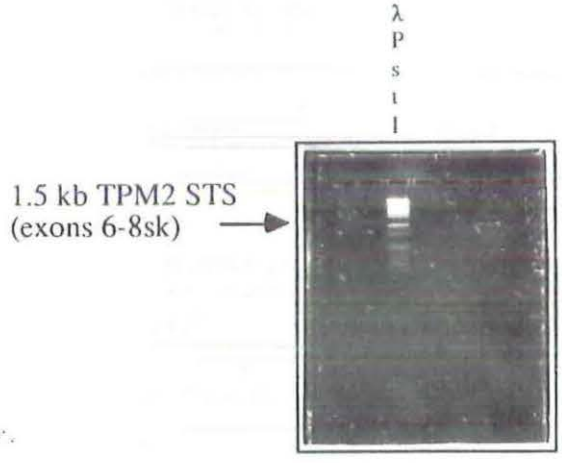
(A)



(B) 2% Low Melting Point Agarose Gel



(C) DNA After Purification



purified DNA concentration of 24 ng/μL matches the faint band seen in Figure 2.7C which was 2 μL (~50 ng) of product run on the gel. The recovery of STS product in the purification process was quite low.

Table 2.3

TPM2 STS DNA Concentration on Spectrophotometer

	Mean A_{260}	Mean A_{280}	A_{260}/A_{280}	Dilution	Concentration (ng/μL)
TPM2 STS 1.5 kb	0.0095	0.0078	1.22	50X	24

4 μL of this template (ie. ~ 100 ng) was used in the cycle sequencing reaction. Primers DS 869 exon 6 and DS 866 exon 8sk were used to sequence in both directions to verify that the STS was indeed from TPM2 human skeletal muscle. The sequencing results extended for about 450 bases from one primer (exon 6 forward, see Figure 2.8) and about 350 bases from the reverse primer (exon 8sk reverse, see Figure, 2.9). From primer DS 869 in exon 6, sequence was obtained from exon 6, all of intron 6, exon 7 and approximately the first 150 bases of intron 7. When the sequencing results were compared with the TPM2 sequence of Widada et al. (1988), out of 70 bases in exon 7 only three bases were different as they could not be read (ie. N). The sequencing in exon 7 was 96% similar to Widada et al.'s TPM2 sequence (see Table 2.4A). The STS and other TPM bases that were different to the TPM2 sequence of Widada et al., are underlined. The eight bolded bases in the STS were unique only to TPM2. Therefore, one can be confident that the 1.5 kb STS was unique to TPM2.

The sequencing results from the reverse primer (DS 866.8R) in exon 8sk, was not as good. The sequence was only readable for 350 bases and included exon 8sk going into intron 7. Out of 48 readable bases for exon 8sk, eight bases differed from the published

FIGURE 2.8

Sequence of TPM 1.5 kb STS from Primer DS 869.6F (Exon 6 Forward)

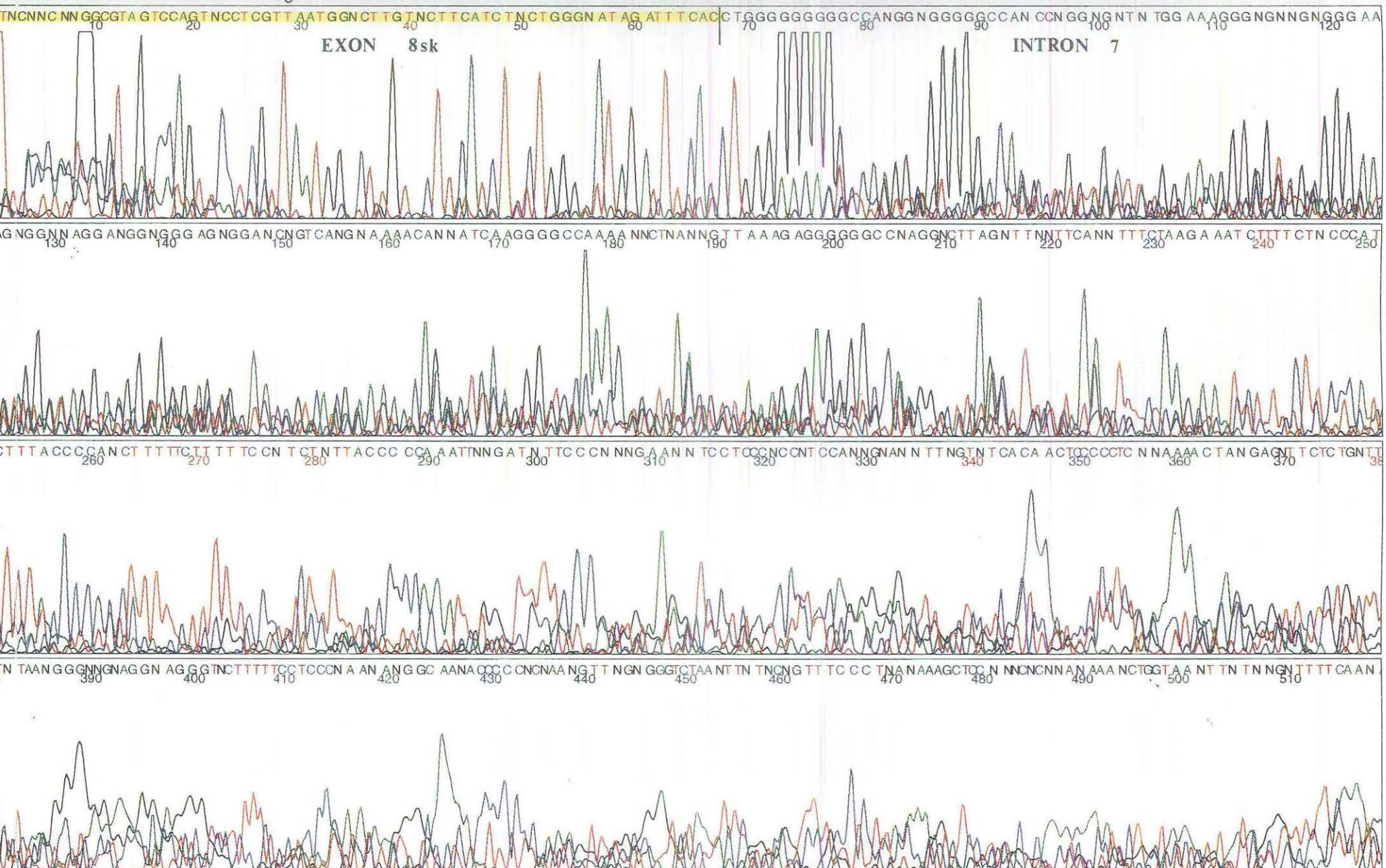

EXON 8sk
INTRON 7

FIGURE 2.9

Sequence of TPM 1.5 kb STS from Primer DS 866.8R (Exon 8sk Reverse)

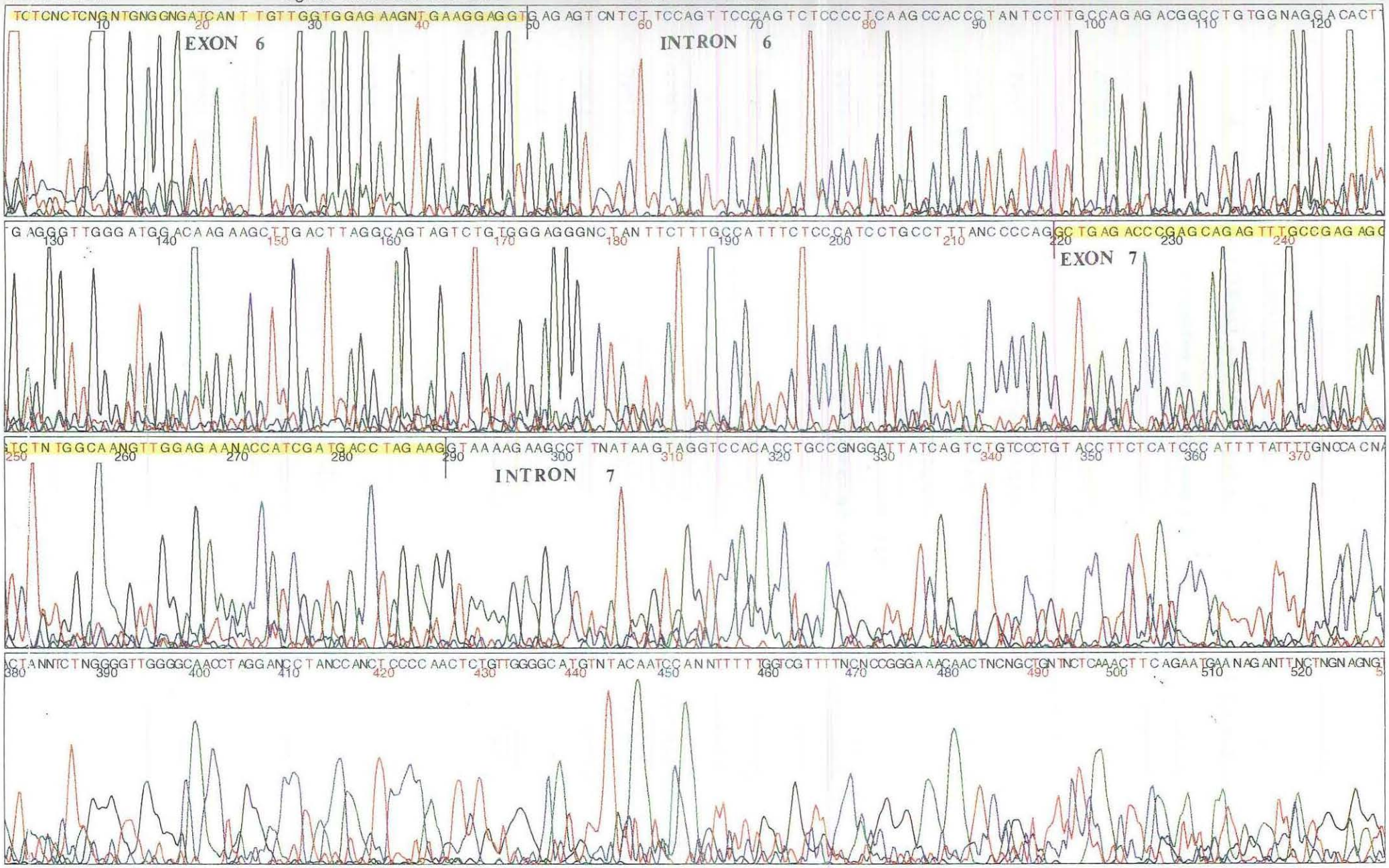

EXON 6
INTRON 6
EXON 7
INTRON 7

Table 2.4

A. Sequence of TPM2 STS from primer DS 869 (exon 6 forward) compared to TPM2 and other TPMs

EXON 6

TPM1	tcctttccga caagctgaag gag
TPM3	ttcttactga taaactcaag gag
TPM4	ttctgtctga caaactgaaa gag
TPM2	tgttggagga gaagctgaag gag
TPM2 STS	t**gt**tggtgga **g**aagntgaag gag

EXON 7

TPM1	gctgagactc gggctgagtt tgcggagagg tcagtaacta
TPM3	gcagagaccc gtgctgagtt tgctgagaga tcggtagcca
TPM4	gctgagaccc gtgctgaatt tgcagagaga acggttgcaa
TPM2	gctgagaccc gagcagagtt tgccgagagg tctgtggcaa
TPM2 STS	gctgagaccc g**a**gcagagtt tgc**c**gagagg tct**g**ntggcaa

TPM1	aattggagaa aagcattgat gacttagaag
TPM3	agctggaaaa gacaattgat gacctggaag
TPM4	aactggaaaa gacaattgat gacctggaag
TPM2	agttggagaa aaccatcgat gacctagaag
TPM2 STS	ngttggagaa naccatcgat gacctagaag

B. Sequence of TPM2 STS from primer DS 866 (exon 8sk reverse) compared to TPM2 and other TPM's

EXON 8sk

```
TPM1          acgagctgta cgctcagaaa ctgaagtaca aagccattag
TPM3          atgagctcta tgcccagaaa ctgaagtaca aggccattag
TPM2          atgaagtcta tgcccagaag atgaagtaca aggccattag
TPM2 STS      gtgaaatcta tncccagnag atgaagnaca agnccattaa

TPM1          cgaggaactg gacaacgc
TPM3          cgaggagctg gaccacgc
TPM2          cgaggaactg gacaacgc
TPM2 STS      cgaggnactg gactacgc
```

sequence of Widada et al., (1988) (see Table 2.4B). The two bolded bases for the STS were unique to TPM2.

Sequencing of the 1.5 kb TPM STS was also performed by ANRI. From the forward primer (DS 869.F) the sequencing results in exon 6 and 7 had only one out of 104 bases which was different to Widada et al.'s published sequence (an N where the sequencer could not distinguish a base). This also confirmed that the STS was specific to TPM2. The 1.5 kb purified TPM2 STS was radiolabelled and used as a probe on a genomic blot (see Figure 2.10). The efficiency of the labelling after the random priming reaction using the PEI strip was approximately 50%. The actual blot membrane had two digests on it. Lanes 1-6 and 8 was total human genomic DNA that was digested with the restriction enzyme *Bam*H I. Lanes 9-16 was human genomic DNA that was cut with

FIGURE 2.10

Radiolabelled 1.5 kb TPM2 STS Used as a Probe on a Genomic Blot

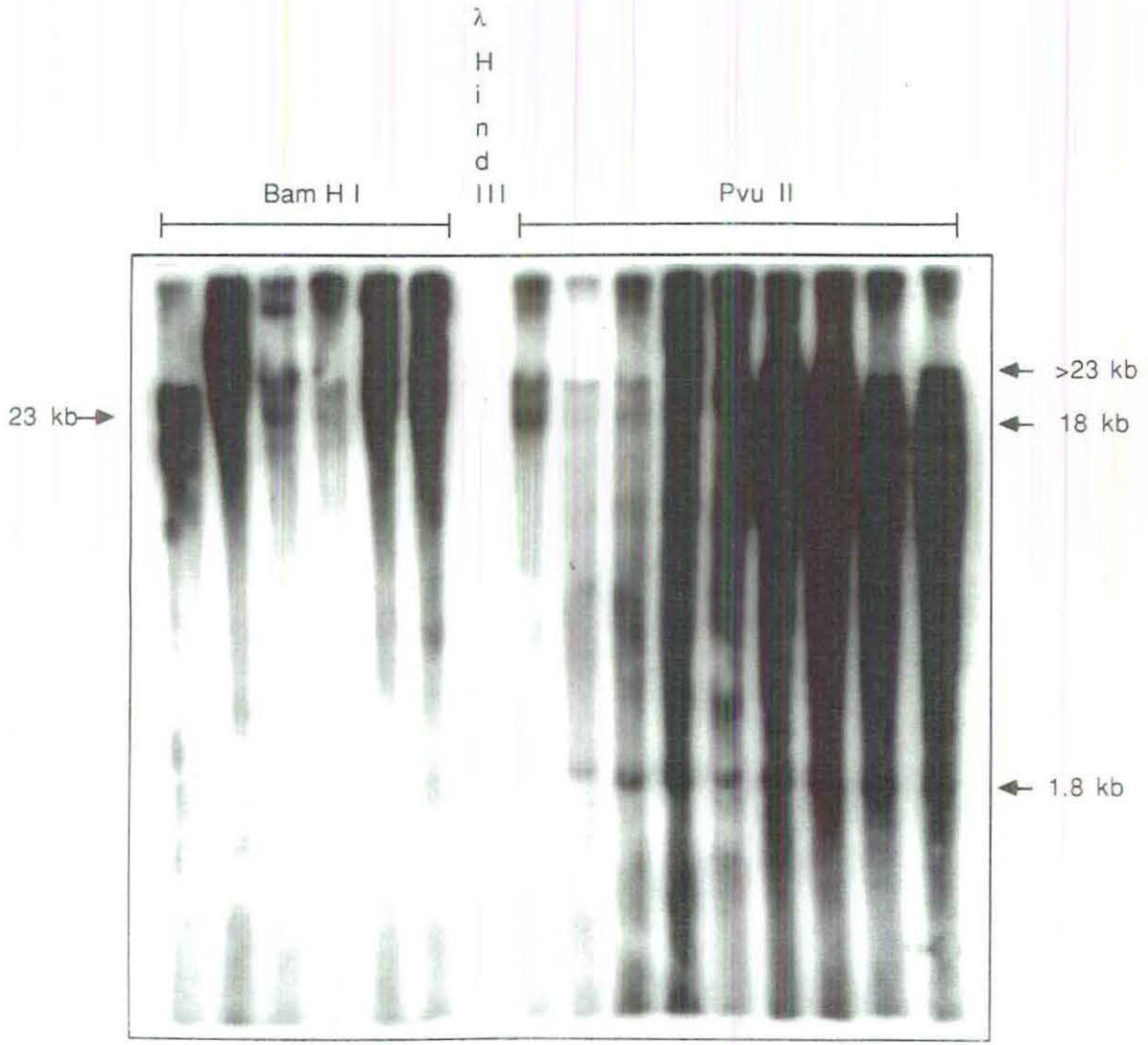
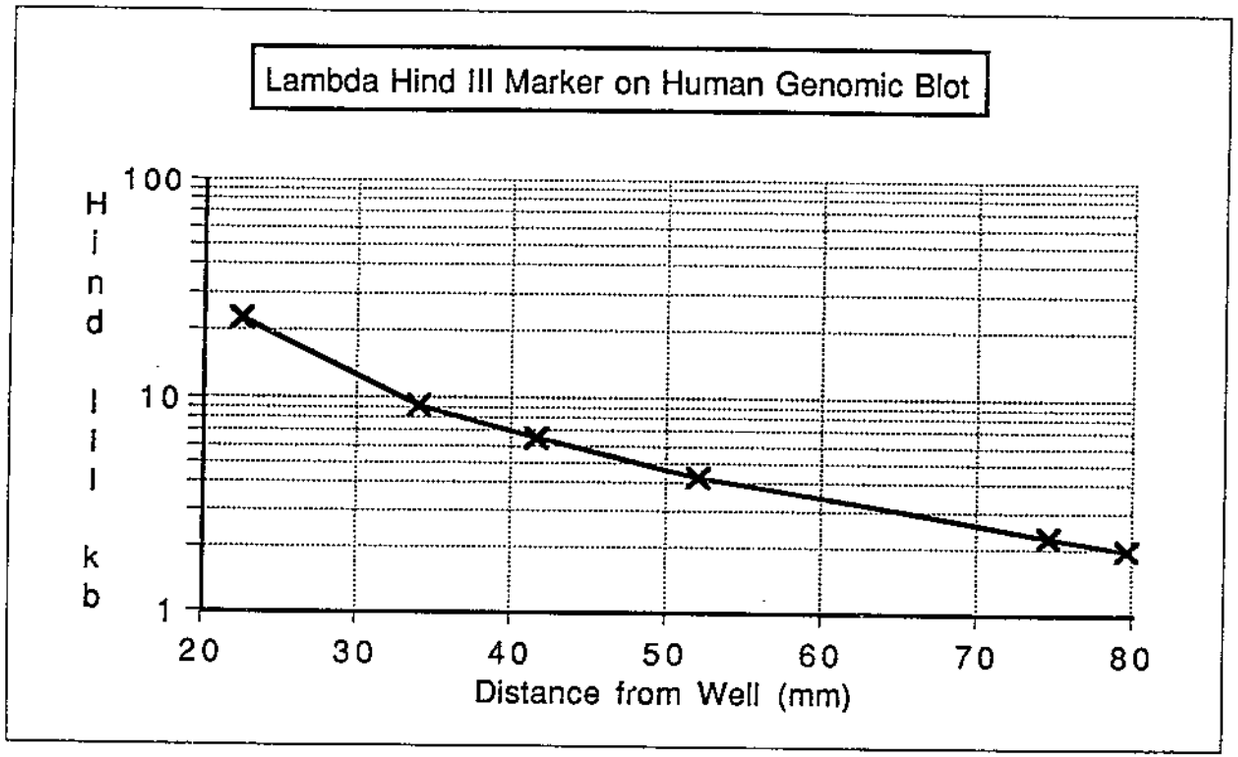

λ
H
i
n
d
III
Bam H I
Pvu II
23 kb
>23 kb
18 kb
1.8 kb

FIGURE 2.11

Distances of Lambda Hind III Marker Fragments from the Well to Compare with the Autoradiograph in Figure 2.12


Lambda Hind III Marker on Human Genomic Blot
Hind III kb
100
10
1
20
30
40
50
60
70
80
Distance from Well (mm)

Pvu II. Lane 7 was the λ*Hind* III marker and the different sized fragments in bp from the well can be seen in Figure 2.11. These were determined from a photograph of the digests when they were run on a gel by ANRI.

One faint band can be seen on the *Bam*H I digest at 23 mm or approximately 23 kb (see Figures 2.10 & 2.11). Three bands can be seen on the *Pvu* II digest. Two faint bands can be seen at 17 mm or greater than the 23 kb λ*Hind* III fragment and 25 mm or around 18 kb. A strong band was seen at 87 mm and represents an approximate 1.8 kb fragment. Most lanes had a lot of non-specific smearing.

2.4 DISCUSSION

The only TPM2 primer combination in Table 2.1 that produced an STS were primers DS 869 (exon 6) and DS 866 (exon 8sk) which amplified an approximate 1.4 - 1.6 kb (average 1.5 kb) product (see Figure 2.7A). The expected STS size from Figure 1.3 and Table 2.2, using the gene structure for TPM3 (Clayton, et al., 1988), was approximately 1.4 kb. Therefore, the amplified 1.5 kb PCR product was close to the expected size and contained introns 6 and 7.

Because only one band was visible in Figure 2.7A, then there were no pseudogenes amplified. If pseudogenes were amplified it would be expected that more than one band would be visible when amplifying genomic DNA. A pseudogene that contained no introns 6 and 7 would have an expected size of 200 bp, but this was not observed.

Sequencing the TPM2 1.5 kb PCR product did confirm that the STS was unique to human skeletal muscle TPM2. Sequencing confirmed two introns (6 and 7) were included in the amplified STS. It was the first time intron 6 had been sequenced. From the reverse primer (DS 866.R in exon 8sk) sequence was obtained from exon 8sk and into intron 7, but it did not reach exon 7 after approximately 450 bases. In TPM3 intron 7 is about 1 kb (Figure 1.3) and it would seem TPM2 is comparable to this.

The sequence differed from the other TPM genes (see Table 2.4 and Appendix 1). This meant the TPM2 STS could confidently be used as a probe for FISH and to isolate a larger clone from a genomic library. The TPM2 STS can also be used on a somatic cell hybrid panel as the sequence was unique.

The 1.5 kb radiolabelled TPM2 STS used as a probe on a genomic blot (Figure 2.10) showed some bands where it was annealing the human genomic DNA. There was also a lot of non-specific binding. The purpose of the TPM2 STS hybridisation to a genomic blot was to see if it hybridised well to genomic DNA, for use in FISH. From the results there was some hybridisation, but the bands were not as defined and clear as it was hoped.

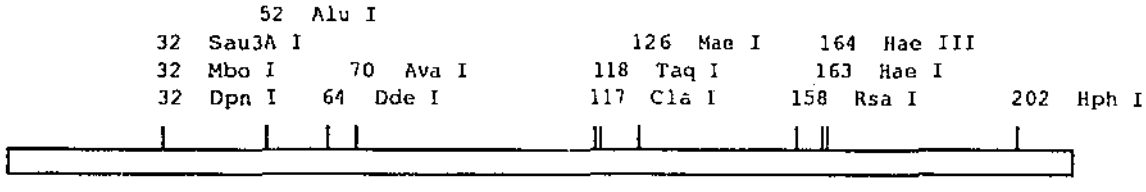
The TPM2 exon sequence from primers DS 869.6F in exon 6, to DS 870.8skR in exon 8sk (see Table 2.4 and Appendix 1) was typed into the application package DNA Strider version 1.0. The restriction map of the cDNA between these two primers is given in Figure 2.12. As can be seen in Figure 2.12, there were no *Bam*H I and *Pvu* II restriction sites in these exons, so the bands from the genomic blot in Figure 2.10 must have been from restriction sites present in the approximate 1.3 kb of intronic sequence.

FIGURE 2.12

Graphic Map of Restriction Sites in TPM2 Exon Sequence from Exons 6-8sk (cDNA) 212 bp.

TPM2 Exons 6-8sk (cDNA) -> Graphic Map

DNA sequence 212 b.p. attccaccaaag ... atcacctcccctc linear



TPM2 Exons 6-8sk (cDNA) 212 base pairs Unique Sites

CHAPTER 3

USING THE TPM2 STS IN A SOMATIC CELL HYBRID PANEL

3.1 INTRODUCTION

A somatic cell is any cell of an organism not involved in the germ line. Somatic cell hybrids are made from fusing together cells from different species. For human gene mapping, human and rodent cells hybrids are commonly used, such as fusing together human and mouse, or human and hamster cells. These somatic cell hybrids can be used for human gene mapping. After several generations, a cell line becomes fairly stable maintaining a specific set of human chromosomes, or a part of the human genome (Gelehrter & Collins, 1990). Human STS markers used on the somatic cell hybrid panel can localise the STS markers.

As long as the panel is representative of the human genome, then performing a PCR of the panel using the human skeletal muscle specific TPM2 primers, should amplify the STS for cell lines containing that part of the TPM2 gene and therefore localise the gene (Dubois & Naylor, 1992; Gelehrter & Collins, 1990).

All work on the somatic cell hybrids was performed by Shelly Dorosz (a research assistant at ANRI) except for the PCR optimisation conditions for the 1.5 kb TPM2 STS, which was supplied by work done in section 2.2.2. The somatic cell hybrid work is described here as it relates to localising the TPM2 gene.

3.2 Materials and Methods

A panel of rodent-human somatic cell hybrids was obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository [NIGMS/mapping panel #2]. The construction of the mouse-human hybrid cell lines was described by Dubois & Naylor (1992) (see Table 3.1). Amplification by PCR was performed with 50 ng of hybrid DNA and the TPM2 STS primers DS 869.6F and DS 866.8skR as in 2.2.2-4. The PCR and amplification conditions used, were those that had been optimised in 2.2.2.

3.3 RESULTS AND DISCUSSION

Table 3.1 shows the percentage of a particular chromosome(s) in each of the hybrid cell lines as determined by both cytogenetic and Southern blot analyses. The panel of 24 hybrid cells is on the most part mono-chromosomal, except GM/NA07299 which contains chromosomes 1 and X, and GM/NA10478 which contains chromosomes 4 and 20 (Dubois & Naylor, 1992).

Initially it was difficult to obtain the 1.5 kb TPM2 STS, however a product was obtained when the amplification conditions that had been optimised in 2.2.2, were used (see Figure 3.1). The TPM2 STS gave an amplification product from only the control human genomic DNA (α & δ) and the somatic cell hybrid GM/NA10611 (n) (Dubois et al., 1992). Only chromosome 9 was unique to this cell line (see Table 3.1) indicating that TPM2 is on human chromosome 9. β and γ were mouse and hamster genomic DNA respectively, which showed that the primers were specific to human DNA from these three species.

TABLE 3.1

Cytogenetic Analysis of NIGMS Human/Rodent

Somatic Cell Mapping Panel 2

(After Dubois & Naylor, 1993)

TABLE 3.1

Cytogenetic Analysis of NIGMS Human/Rodent Somatic Cell Mapping Panel 2 (from Camden Literature)

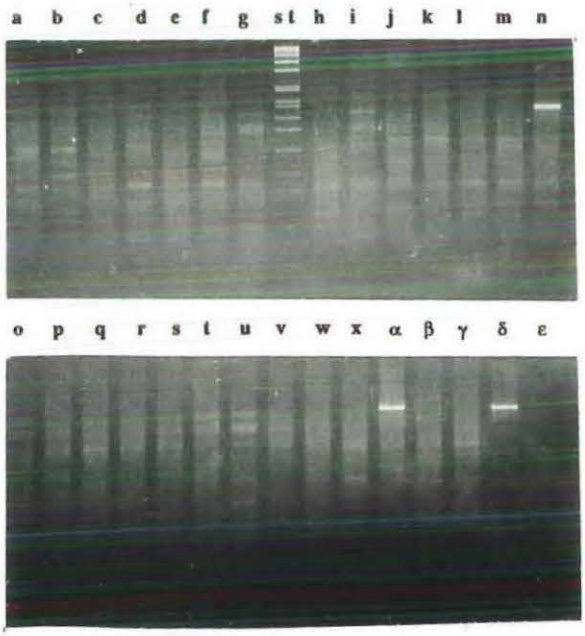
	Chromosome																						
Hybrid/DNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
GM/NA07299	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	85	0
GM/NA10826B	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10253	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10115	0	0	0	97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10114	0	0	0	0	93	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10529	0	0	0	0	0	98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10791	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10156B	0	0	0	0	0	0	0	86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10611	0	0	0	0	0	0	0	0	69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10926B	0	0	0	0	0	0	0	0	0	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10927A	0	0	0	0	0	0	0	0	0	0	92	0	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10868	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0
GM/NA10898	0	0	0	0	0	0	0	0	0	0	0	0	92	0	0	0	0	0	0	0	0	0	0	0
GM/NA10479	0	0	0	0	0	0	0	0	0	0	0	0	0	88	0	0	0	0	0	0	0	0	0	0
GM/NA11418	0	0	0	0	0	0	0	0	0	0	0	0	0	0	68	0	0	0	0	0	0	0	0	0
GM/NA10567	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	98	0	0	0	0	0	0	0	0
GM/NA10498	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	96	0	0	0	0	0	0	0
GM/NA11010	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	72	0	0	0	0	0	0
GM/NA10449	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	96	0	0	0	0	0
GM/NA10478	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	76	0	0	0	0
GM/NA10323	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	86	0	0	0
GM/NA10888	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0
GM/NA06318B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0
GM/NA06317	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	96

FIGURE 3.1

1.5 kb TPM2 STS vs NIGMS Human Rodent Somatic Cell Hybrid Mapping Panel 2 by PCR

	Chromosome	Hybrid/DNA Cell Line
a	Y	GM/NA06317
b	X	06318B
c	1 & X	7299
d	5	10114
e	4	10115
f	8	10156B
g	3	10253
standard	λ *Pst* I	-
h	21	10323
i	19	10449
j	4 & 20	10478
k	14	10479
l	17	10498
m	16	10567
n	9	10611
o	6	10629
p	7	10791
q	2	10826B
r	12	10868
s	22	10888
t	13	10898
u	10	10926B
v	11	10927B
w	18	11010
x	15	11418
α	Human Genomic	NAIMR91
β	Mouse Line 376	NA05862
γ	Chinese Hamster Line RJ K88	NA10658
δ	Human Genomic	ANRI
ε	Blank	-

TPM2 STS vs HUMAN/RODENT HYBRID PANEL



CHAPTER 4

USING THE TPM2 STS IN FISH

4.1 INTRODUCTION

Fluorescent *in situ* hybridisation (FISH) mapping is where a fluorescently labelled probe hybridises to human metaphase spreads, so its location can be determined.

Cells are grown in culture and treated with colchicine to arrest cells at the metaphase stage of mitosis. Colchicine is believed to prevent the formation of spindle fibres (Biochemica, 1992). The chromosomes remain within the nuclear membrane and are fixed and spread on to a microscope slide. (Davies & Read, 1992).

The probe is labelled by coupling biotin covalently to it. Although probes can be directly conjugated with fluorescent molecules, the most widespread approach is to label the probes with reporter molecules such as biotin or digoxigenin that, after hybridisation, bind fluorescent affinity agents. The reporter molecules are labelled to the probe using nick translation (Trask, 1991).

Nick translation was originally described by (Rigby, et al., 1977). The process is where free 3'-hydroxyl ends or 'nicks' are created within unlabelled double stranded DNA by DNase I. DNA polymerase I then catalyses the addition of a nucleotide to the 3'-hydroxyl terminus of the nick. At the same time, the 5' to 3' exonuclease activity of this enzyme removes the nucleotide from the 5'-phosphoryl terminus of the nick. A new nucleotide with a 3'-OH group is incorporated at the position where the original nucleotide was excised and results in the movement of the nick (nick translation) along the DNA 5' to 3', as new nucleotides labelled with biotin are incorporated (Promega, 1991; Sambrook, et al., 1989). Usually biotinylated nucleotides adenosine and cytosine triphosphates are synthesised for incorporation (Biochemica, 1992). Incorporation can be up to 65%.

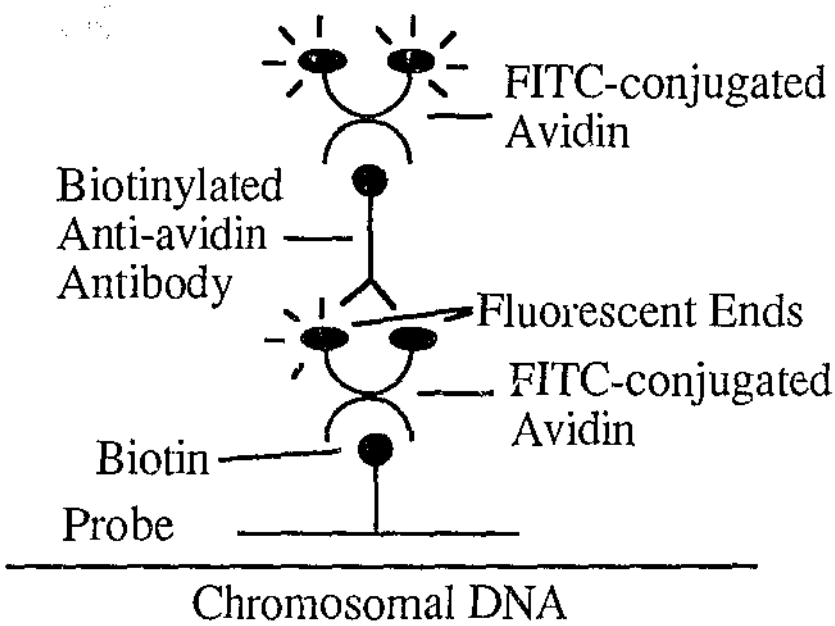
(Promega, 1991). Nick translation reduces the size of the probe after the reaction to 200-500 bp fragments (Biochemica, 1992).

Chromosomes within the nuclear membrane are then denatured by heat or by altering the pH on the slide, without destroying the morphology of the chromosomes (Trask, 1991). Simultaneously, the probe is also denatured by heat or altering the pH. The slide is then incubated with the labelled single-stranded probe which hybridises to any matching sequences in the denatured chromosomes. The accuracy of annealing depends on the stringency of the hybridising conditions. High stringency hybridisation allows only exact complementary strands to rejoin (Davies & Read, 1992; Leversha, 1993). Any unbound probe is then washed off the slide at increasing stringencies. Probe segments which bind to chromosomal sequences with the greatest similarity (or homology) will withstand more stringent washing than those which happen to bind to less closely matched sequences (Leversha, 1993).

After washing, the slides are incubated in immunofluorescent reagents to produce a fluorescent signal at the sites of biotin-labelled probe hybridisation (Trask, 1991). The signal is developed using a streptavidin or avidin-carrying fluorescent reporter molecule that binds to the biotin. One or more layers of antibodies may be included to amplify the signal. The antibodies bind to the avidin and then allow more avidin-carrying fluorescent molecules to bind to the antibody. With successive layers of antibody and avidin, the signal is amplified (see Figure 4.1) (Davies & Read, 1992; Leversha, 1993). Avidin or specific antibodies can also be linked to fluorochromes such as fluorescein isothiocyanate (FITC) or Texas Red. The biotin labelled probe is detected with immunochemicals linked to different fluorochromes such as FITC-conjugated Avidin in Figure 4.1 (Leversha, 1993).

FIGURE 4.1

Biotin-Avidin Detection Systems for Fluorescence *In Situ* Hybridisation (FISH)



(After Leversha, 1993)

The chromosomes are then stained with propidium iodide (red), as a counterstain to outline the chromosomes and 4', 6-diamidino-2-phenylimdole DAPI (blue), which gives a pattern of dark and light G-bands (the same as Giemsa banding) for chromosomal identification (Biochemica, 1992; Trask, 1991).

The FITC signal (494 nm maximum excitation yellow) can be detected by a range of fluorescent microscopy equipment. One of the latest and most sensitive systems is digital imaging microscopy, where a cooled, charged coupled device (CCD) camera counts emitted photons over a broad spectrum. The signal is computer enhanced to give a two-dimensional analysis (Biochemica, 1992).

On metaphase spreads, FISH resolution is 10-20 million bp, which is 0.3-0.6% of the genome or 8-16% of an average chromosome (Davies & Read, 1992).

In interphase nuclei, fluorescent dots can be seen by FISH. These cannot reveal the chromosomal location, but if sets of probes with different fluorescent labels are used, their distance apart and their order can be established with a resolution as precise as 40 kb (Davies & Read, 1992).

If the TPM2 STS probe is larger than 1 kb then it can be used for fluorescent *in situ* hybridisation (FISH). FISH was carried out on a metaphase spread of normal human male chromosomes. Male chromosomes are used because they have both the X and Y chromosomes, so every chromosome is included. The STS will anneal to a chromosome at a particular band where the gene will be localised. FISH was carried out at the Adelaide Children's Hospital by Helen Eyre and Dr David Callen, as the techniques require considerable expertise, beyond the scope of this project (Akkari, et al., 1994; Eyre, et al., 1993).

4.2 MATERIALS AND METHODS

The TPM2 STS was larger than 1 kb (1.5 kb) so it was used for fluorescent *in situ* hybridisation (FISH).

The probes were nick-translated with biotin-14-dATP and each was hybridised *in situ* at a final concentration of 20 ng/µl to metaphases from two normal males. The fluorescence *in situ* hybridisation (FISH) method was modified from that previously described (Callen, et al., 1990). Only two rounds of amplification with fluorescein-conjugated avidin and biotinylated goat anti-avidin were required. The chromosomes were stained before analysis with both propidium iodide (as counterstain) and (DAPI) (for chromosome identification). Images of metaphase preparations were captured by a CCD camera and computer enhanced (Callen, et al., 1992; Eyre, et al., 1993).

4.3 RESULTS AND DISCUSSION

The 1.5 kb TPM2 STS yielded unspecific hybridisation results of this probe to the metaphase spreads from two normal males. The probable cause was that the probe did not have enough sequence, as usually a minimum of 1 kb is required for FISH (Akkari, et al., 1994) and the TPM2 probe was only slightly longer than the minimum required.

Two approaches could have been taken from here. The first would have been to try to amplify a longer TPM2 STS for FISH, but a range of primers had already been tried unsuccessfully. The maximum expected STS size with conventional PCR techniques is around 4-6 kb (Gelehrter & Collins, 1990; Hayashi, 1994; Saiki, 1989) and there was no guarantee another PCR STS would work for FISH. The second approach was to use the STS as a labelled probe to isolate a larger genomic clone from a genomic library for use in FISH. In bacteriophage, genomic clones have human inserts of between 8-21 kb that would have more DNA sequence to hybridise to the human metaphase spread and so give a stronger labelled signal (Clontech, 1994)

CHAPTER 5

ISOLATION OF A CLONE FROM A GENOMIC LIBRARY

5.1 INTRODUCTION

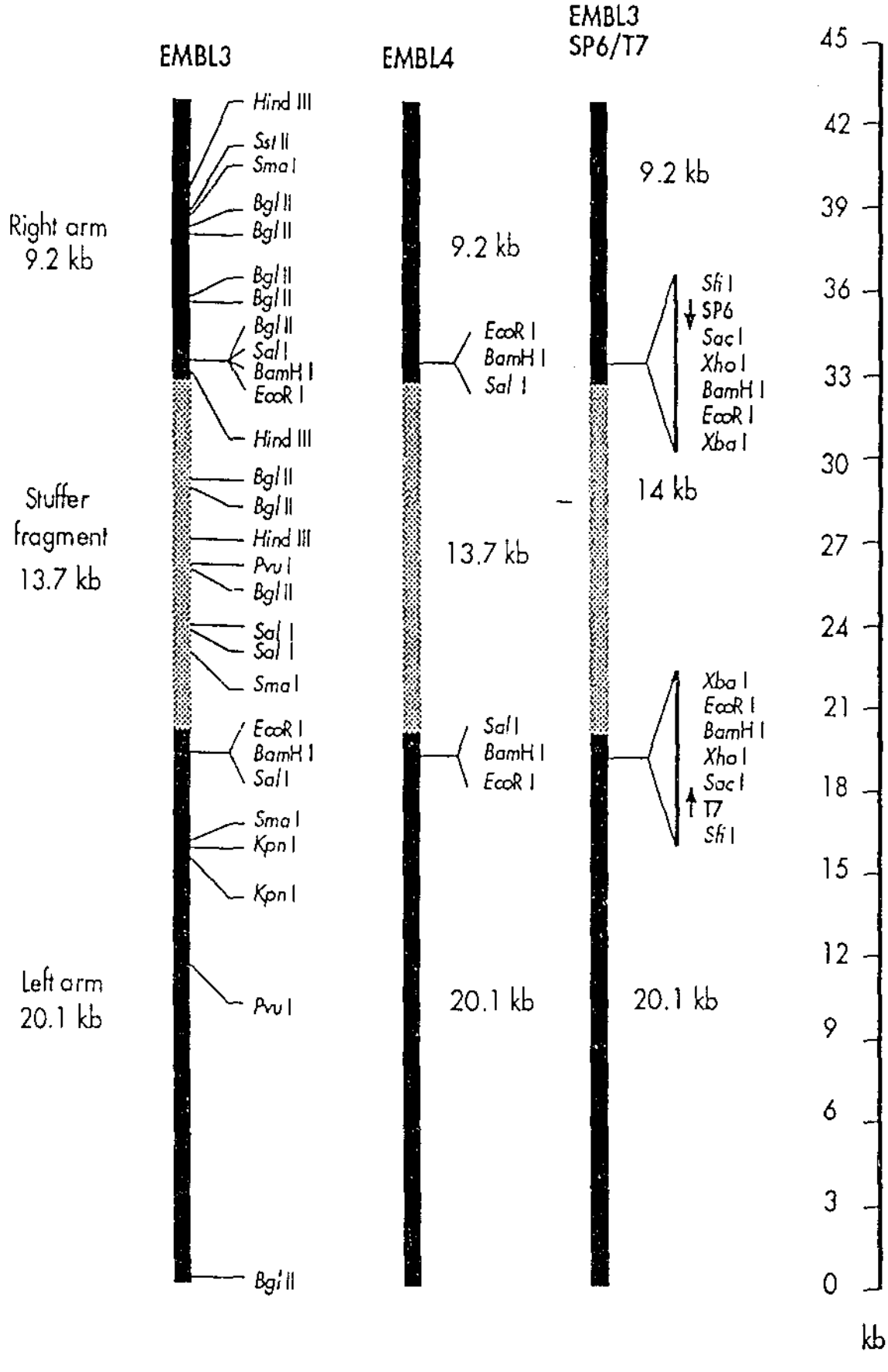
The radiolabelled 1.5 kb TPM2 STS was used as a probe to isolate a clone from a genomic library. The clone would contain a larger portion of the TPM2 gene and perhaps the flanking region for use in FISH. The genomic library was constructed in EMBL3 SP6/T7 (Clontech). The DNA was partially digested with *Sau*3A I and the fragments were separated on a sucrose gradient to produce a size range between 8 and 22 kb before cloning into the *Bam*H I site of EMBL3 SP6/T7. The average insert size was 15 kb. The insert DNA could be excised with *Xho* I, *Sac* I, or *Sfi* I (see Figure 5.1) (Clontech, 1994). The human insert replaces the stuffer fragment or central portion of phage DNA. The stuffer region is not essential for phage growth and reproduction.

The supplied host strain was *Escherichia coli* K802 (Clontech, 1994). Bacteriophage adsorb to receptors in the outer membrane of *E. coli* that are encoded by the bacterial *lamB* gene which are normally used to transport maltose into the cell. When culturing the host strain on an agar medium, maltose must be included as it induces the synthesis of these receptors, so the host strain can be infected by the bacteriophage. Adsorption of bacteriophage to maltose receptors is facilitated by magnesium ions and occurs within a few minutes at room temperature and at 37°C. The bacteriophage DNA is inserted through the cell membrane and undergoes lytic growth. The genome of bacteriophage is double stranded linear DNA, approximately 45 kb long. After infection, the linear DNA forms a closed circular molecule that serves as the template for transcription. During lytic growth, the circular DNA is replicated many times and a large number of bacteriophage gene products are synthesised.

FIGURE 5.1

EMBL3, EMBL4 & EMBL3 SP6/T7 Vector Maps

(After Clontech, 1994)


EMBL3
EMBL4
EMBL3
SP6/T7
Right arm
9.2 kb
Stuffer
fragment
13.7 kb
Left arm
20.1 kb
Hind III
Sst II
Sma I
Bgl II
Bgl II
Bgl II
Bgl II
Bgl II
Sal I
BamH I
EcoR I
Hind III
Bgl II
Bgl II
Hind III
Pvu I
Bgl II
Sal I
Sal I
Sma I
EcoR I
BamH I
Sal I
Sma I
Kpn I
Kpn I
Pvu I
Bgl II
9.2 kb
EcoR I
BamH I
Sal I
13.7 kb
Sal I
BamH I
EcoR I
20.1 kb
9.2 kb
Sfi I
SP6
Sac I
Xho I
BamH I
EcoR I
Xba I
14 kb
Xba I
EcoR I
BamH I
Xho I
Sac I
T7
Sfi I
20.1 kb
45
42
39
36
33
30
27
24
21
18
15
12
9
6
3
0
kb

Progeny phage particles are assembled and the cell eventually lyses, releasing hundreds of new infectious viruses (Clarke & Carbon, 1976; Hayes, 1968; Primrose & Dimmock, 1980; Sambrook, et al., 1989). This process from infection to lysis takes approximately 15 min at 37°C (Clontech, 1994).

The EMBL3 SP6/T7 vector is a virulent phage so it only undergoes the lytic cycle. The phage are plated out by incubating a relatively small number of phage particles (1-100 000) to a large number of concentrated bacteria (eg. $100x10^6$) so that it is likely each bacterium is infected by only one phage. The mixture is poured over a nutrient agar plate and incubated so the uninfected bacteria forms a confluent film over the plate. Each infected bacterium, lyses after a short time releasing several hundred progeny phages which then infect adjacent bacteria, which in turn are lysed. This process spreads locally through the bacterial population until the bacterial population reaches the stationary phase where no more bacteria multiply and bacterial metabolism declines. The result is a visible circumscribed area of clearing in the bacterial growth, known as a plaque (Fraser, 1967; Hayes, 1968; Primrose & Dimmock, 1980). This plaque is a clone containing only one type of recombinant DNA or human insert. A probe specific to TPM2 was used to isolate plaque(s) or clone(s) that contained a part of the TPM2 gene so it could be amplified and the DNA extracted for use in FISH.

Firstly, though, the 1.5 kb TPM2 STS primers were used on the EMBL3 library, to see if that segment of the TPM2 gene was present in the library.

5.2 MATERIALS AND METHODS

5.2.1 Determining if the 1.5 kb TPM2 STS was in the Library

The 1.5 kb TPM2 STS primers that were developed in chapter 2, were used to determine if that segment of the tropomyosin 2 gene was present in the library (ie. exons 6 - 8sk). A PCR was set up as in chapter 2.2.2 using 1 µL of the neat EMBL3 SP6/T7 library, as

the target DNA, for primers DS 869.6F to DS 866.8skR. 50 ng of total genomic DNA was also used as a positive control. The PCR for each different target DNA was repeated three times to verify the experiment.

Assuming the EMBL3 SP6/T7 vector had an average human DNA insert of 15 kb and that 1 µL of the 'neat' library contains $5x10^6$ pfu/µL (Clontech, 1994) and there are $3x10^9$ bp in the human genome, then:

The No. of human genomes in 1 µL of the EMBL3 SP6/T7 library =
No. pfu/µL x Average No. bp per human insert / No. bp in human genome

The No. of genomes in 1 µL of the EMBL3 SP6/T7 library = $\frac{5x10^6 \text{ pfu/µL} \times 15\,000 \text{ bp}}{3x10^9 \text{ bp}}$

= 25 genomes

Using 1 µL of the EMBL3 SP6/T7 library on average represents 25 genomes of human DNA and should provide enough representative samples of the human genome to amplify the 1.5 kb TPM2 STS, using the primers.

5.2.1.1 Protocol

Reaction Mixture	**1X 20 µL**	**8 X**
Tth Buffer (5.2^{200})	5 µL	40 µL
Primer mix (DS 869 + 866)	2 µL	16 µL
Tth (5.5 u/µL)	0.1 µL	0.8 µL
H_2O	12.9 µL	103.2 µL
Total	20.0 µL	160.0 µL

Lanes 1-3	3X EMBL3 SP6/T7 bacteriophage	1 µL neat and 4 µL H_2O
Lanes 4-6	3X Human genomic DNA (D90-224)	5 µL
Lane 7	1X Blank (H_2O)	5 µL
Lane 8	λ Pst I Marker	3 µL

The 8X reaction mixture was added with the target DNA to make a 25 µL reaction. So 20 µL of the reaction mixture was added to 1 µL EMBL3 SP6/T7 and 4 µL H_2O, and 20 µL of the reaction mixture was added to 5 µL of the human genomic DNA and blank. Cycling conditions and agarose electrophoresis were the same as 2.2.2-4.

5.2.2 Aliquots of the Library

The vector and host stain were stored at -80°C. An aliquot of the EMBL3 SP6/T7 library was made as a backup by aliquoting 200 µL of the neat library using aseptic techniques into a 0.6 mL eppendorf tube and storing it in a different -80°C freezer.

5.2.3 Bacterial Culture Plating and Propagating the Host Strain (K802)

Using aseptic techniques, 5 µL of the host strain K802 was streaked onto a $MgSO_4$ - free 2X YT 1.5% bottom agar plate containing 0.2% maltose. This was incubated at 37°C overnight. From the agar plate a single isolated colony was picked and propagated in 10 mL 2X YT liquid media and 10 mM $MgSO_4$ and 0.2% maltose in a 50 mL polypropylene tube. The broth was incubated at 37°C on a shaker at 200 rpm (B. Braun Model Centomat H), well aerated overnight, for approximately 16 hours, or until the optical density at 600 nm (OD_{600}) reached 2.0, or until the stationary phase of the culture was reached. 850 µL of the broth culture was added to 150 µL of glycerol to make a glycerol stock. Two glycerol stocks were prepared and stored at -20°C. All working samples were taken from the glycerol stocks, while the original host strain was stored at -80°C (Clontech, 1994).

5.2.4 Library Plating/Titering

15 cm petri dishes were used to plate out the EMBL3 SP6/T7 library requiring approximately 50 000 pfu. The library therefore needed to be diluted to yield 50 000 pfu

per plate. Since 1 µL of the library had between 1-9x10^6 pfu/µL then to dilute 1 µL of this library to produce 50 000 pfu per plate required:

$$\frac{\text{1-9x}10^6\ \text{pfu/µL}}{\text{50 000 pfu}} = 180\ \text{µL of 1X lambda dilution buffer (see Appendix 2)}$$

If 1 µL of the neat library contained 25 genomes worth of human DNA then 1 µL of the (1/180X) dilution would have:

$$25/180 = 0.14 \text{ of the human genome}$$

Therefore, to plate out one genome's worth of human DNA requires:

$$1/0.14 = 7.2 \text{ or eight 15 cm plates}$$

The number of clones required (N) to screen to get a particular clone with a probability P.

$$N = \frac{\ln (1-P)}{\ln (1-1/n)}$$

where $n = \frac{\text{genome size (bp)}}{\text{average size of insert (bp)}}$ and P = 0.95, 0.99 etc

(Clarke & Carbon, 1976)

$$N = \frac{\ln (1-0.95)}{\ln (1-15\,000/3\text{x}10^9)} = 600\,000 \text{ clones}$$

If each 15 cm petri dish has 50 000 pfu, then the number of plates required to plate out the library to 95% certainty that at least one clone will contain part of the TPM2 gene is:

$$600\,000/50\,000 = 12 \text{ plates}$$

However, it was decided to plate out at least one genome's worth of the genomic library (8 plates) at first, to see if the radiolabelled TPM2 STS would at least give a positive signal to some clones.

Using 8 plates of 50 000 pfu/plate, then rearranging the formula, the probability that at least one plaque will contain part of the TPM2 gene is:

$$P = 1 - e^{(N.\ln(1-1/n)}$$

$$P = 1 - e^{(400\,000.\ln(1-15\,000/3x10^9)}$$

$$P = 0.86 \text{ or } 86\%$$

1. Eight 15 cm 2X YT 1.5% agar plates were poured having 10 mM $MgSO_4$ and 0.2% maltose concentration. Using aseptic techniques, enough agar was poured to cover the bottom of a 15 cm diameter petri dish (~30 mL). When necessary, the top of the agar was flamed to remove bubbles and then left to set for approximately 15 min.

2. A 10 mL 2X YT broth (+10 mM $MgSO_4$ and 0.2% maltose) was inoculated with the host strain (K802) from the glycerol stock. It was incubated on a shaker (200 rpm) at 37°C overnight.

3. To each of eight 50 mL polypropylene tubes was added 200 µL of the overnight broth inoculated with 1 µL of the diluted (1/180X) EMBL3 SP6/T7 library and re-incubated at 37°C for 20 min.

4. After 20 min 10 mL of melted (~50°C) top 0.7% agarose (+10 mM $MgSO_4$ and 0.2% maltose) was added to each of the eight tubes and mixed well.

5. The contents of each tube was poured on to the eight separate 2X YT 1.5% bottom agar plates (+10 mM $MgSO_4$ and 0.2% maltose). The plates were quickly swirled to allow even spreading of the agarose. Any bubbles were removed with a flame.

6. The plates were cooled to room temperature for 10 min to allow the inoculum to soak into the agar.

7. The plates were inverted to prevent contamination and incubated at 37°C overnight or until the plaques reached a diameter not exceeding 1.5 mm, or were just beginning to make contact with one another, near confluency (~8-16 hours). Confluent lysis was deliberately avoided.

8. The plates were sealed in parafilm and stored at 4°C.

(Clontech, 1994; Sambrook, et al., 1989).

5.2.5 Membrane Replicas of the Agar Plates

1. A 137 mm diameter nylon nucleic acid transfer membrane (Hybond-N^+, Amersham) was labelled with a ball point pen. Using sterile forceps, the membrane was placed onto the 2X YT 0.7% top agarose. Trapping air bubbles was avoided. The membrane was marked in 3 or 4 asymmetrical locations by stabbing through the membrane and into the agar with an 18-gauge needle that was dipped into printer's black ink (see Figure 5.2).

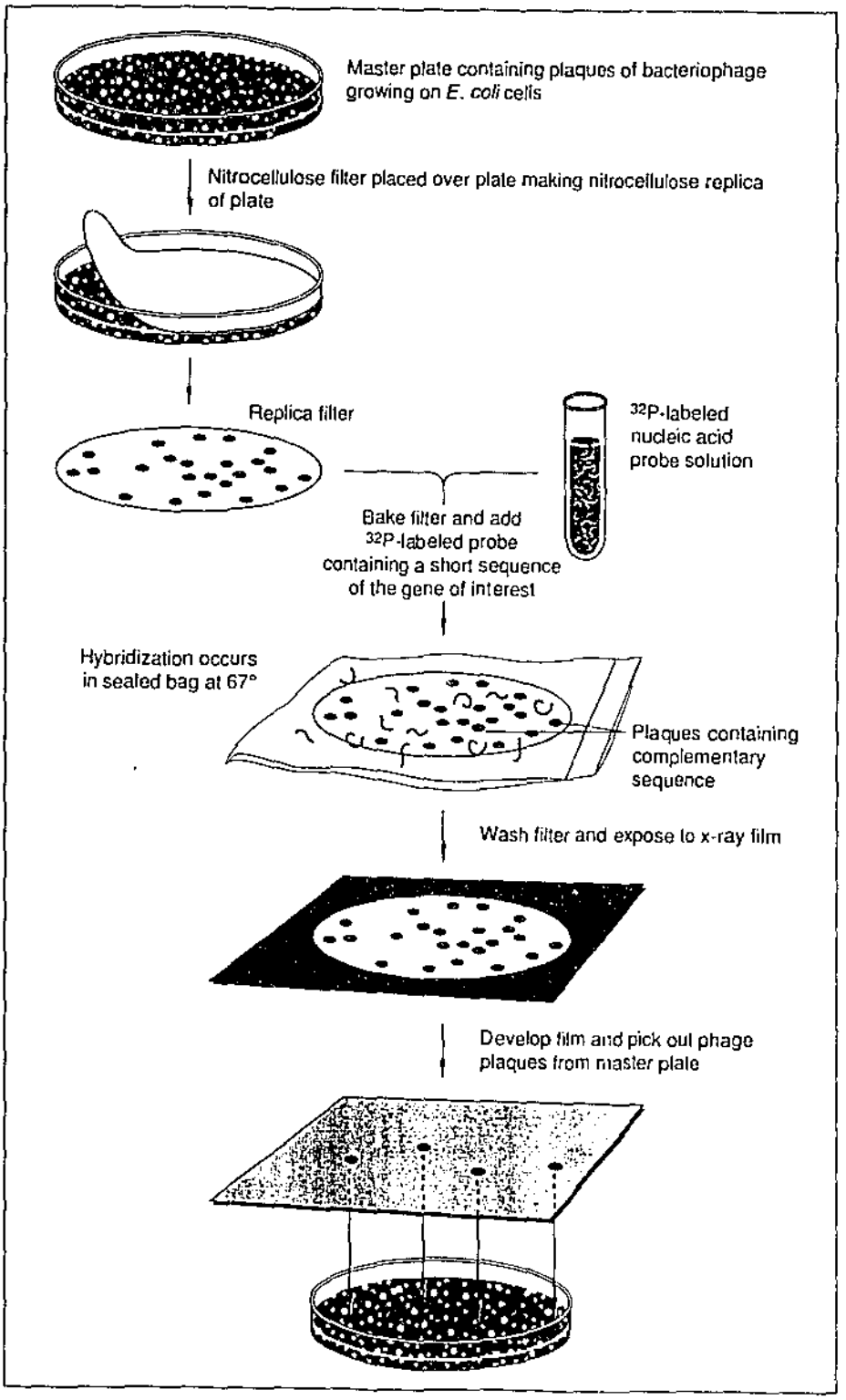
2. After 2 min, the membrane was carefully peeled off. The filter was placed plaque side up, onto a 15 x 15 cm Whatman 3MM chromatography paper soaked in NaOH denaturing solution (see Appendix 2) for 2 min.

3. The membrane was then placed plaque side up on another 15 x 15 cm Whatman 3MM paper soaked in the Tris neutralising solution (see Appendix 2) for 2 min.

FIGURE 5.2

Screening a Genomic Library

(After Deaven, 1994)


Master plate containing plaques of bacteriophage growing on *E. coli* cells
Nitrocellulose filter placed over plate making nitrocellulose replica of plate
Replica filter
^{32}P-labeled nucleic acid probe solution
Bake filter and add ^{32}P-labeled probe containing a short sequence of the gene of interest
Hybridization occurs in sealed bag at 67°
Plaques containing complementary sequence
Wash filter and expose to x-ray film
Develop film and pick out phage plaques from master plate

4. The membrane was then placed between two Whatman 3MM papers to blot dry.

5. A second Hybond N^+ membrane (the duplicate lift) was placed onto the same plate and marked with ink in the same locations. The membrane was peeled off after 2 min and denatured and neutralised on the same Whatman 3MM papers as described in Steps 2-4.

6. The same procedure was used for lifting the other plates, except the membranes were denatured and neutralised on freshly soaked Whatman 3MM papers.

7. The membranes were left to dry, at room temperature, overnight in a dark place, stacked between Whatman 3M papers.

(Clontech, 1994; Sambrook, et al., 1989)

5.2.6 Hybridisation of the Membrane Replicas using the Radiolabelled 1.5 kb TPM2 STS

1. The membranes were placed in a round plastic container with prehybridisation solution and incubated at 42°C for at least four hours shaking (Stuart Scientific Hybridisation Oven/Shaker) (as in section 2.2.9.4). 10 mL of prehybridisation solution was added per membrane.

2. The randomly primed 1.5 kb TPM2 STS (sections 2.2.9.2 - 2.2.9.6) was denatured by heating to 95°C and snap chilled on ice. 50 µL of the probe was then added to the prehybridised membranes and incubated at 42°C for between 16-20 hrs.

3. The membranes were removed and washed at increasing stringency until the cpm were between 10-20 cpm (section 2.2.9.7).

4. The membranes were blot-dried on Whatman 3M paper and wrapped in Glad Wrap. The duplicate membranes were paired and placed with tape to a large piece of Whatman

3MM paper as backing paper, that was cut to fit the size of the autoradiograph cassette, so the membranes would not move. The Whatman paper was marked in three asymmetrical places with a fluorescent dye. The membranes were placed in a cassette for autoradiography. Autoradiography was performed with Dupont Cronex 4 X-ray film and Quanta III autoradiography intensifying screens, at -80°C for an empirically determined time, usually overnight.

5. After developing, the film was aligned with the membranes to make sure they did not move. The film was then aligned with the agar plates to select a positive plaque that was present on the film from the two duplicate lifts. A single isolated plaque was picked, but in cases where the plaques were becoming confluent, an agar plug was removed with several plaques (only for the initial screening).

6. A plaque was picked by cutting off the end of a disposable pipette tip on a 200 µL pipette. The tip was pushed through the 0.7% agarose top and bottom agar where a positive plaque was selected and the agar plug was removed by the suction of the pipette. The agar plug was placed into a sterile 1.5 mL eppendorf with 200 µL of 1X lambda dilution buffer, so the phage eluted into solution. The agar plugs were stored at 4°C.

7. Using the eluted phage from each selected positive plaque, the phage were replated (called secondary replating) and titered to obtain 200-1000 plaques on a 15 cm petri dish. This was achieved by using 1, 10, and 100 µL of the eluted phage from the agar plug in 200 µL of the overnight broth, to obtain the optimum titre on a plate.

8. The optimum titered plates were lifted using the nylon membranes (called secondary lifts) and rescreened using the radiolabelled 1.5 kb TPM2 STS (called secondary re-screening). The procedure was repeated until 100% of the plaques showed up as positive. This would require rescreening three or four times for each different primary plaque clone that was selected from the initial screening.

9. For each clone a well isolated positive plaque was selected for plate stock. This was to grow the clone on many plates to confluency, so the DNA could eventually be extracted and purified for use in FISH.

(Clontech, 1994; Sambrook, et al., 1989)

5.2.7 PCR assay of DNA from Clone Eluates to Confirm Clones Contain Part of the TPM2 Gene.

Isolated clones of the final screening were picked. Primers DS 869 (exon 6F) and DS 866 (exon 8sk.R) were used with the final picked clones to determine if the human inserts contained that part of the TPM2 gene. A 25 µL PCR was set up as in 5.2.1.1 and 2.2.2-4 with 5 µL of the clones eluates used as the template DNA. 5 µL of human genomic DNA was used as a positive control and a blank was run as a negative control. If the 1.5 kb TPM2 STS showed up as a band from the eluates, it meant the clones contained that part of the TPM2 gene (exons 6-8sk) and that they could be plated out in mass, to isolate the DNA from phage lysates.

5.2.8 Isolation of DNA from Phage Lysates

5.2.8.1 Preparation of bacterial host (K802) cells

0.5 µL of the glycerol stock of K802 was incubated in 10 mL of 2X YT broth, 10 mM $MgSO_4$ and 0.2% maltose. This was left shaking (200 rpm), at 37°C overnight.

5.2.8.2 High-titre phage stock preparation

The ratio of phage to bacterial host, determines the success of the phage DNA preparation. This must be determined empirically. A yield of 20-40 µg of phage DNA could be expected from 100 mL of phage lysate (Clontech, 1994).

1. A selected clone, was titered using the eluted phage from the agar plug (1, 10 and 100 µL).

2. 10 mL of 1X lambda dilution buffer and a few drops of chloroform were added to the plates that had a titre of higher than 100 pfu. These plates were left shaking overnight at 4°C to elute the phage. The chloroform lyses the bacterial cell walls so the phage were more easily eluted.

3. The eluted phage from the plates was pooled and poured into a 50 mL Oakridge tube.

4. 2 mL of chloroform was added to the lysate and vortexed for 2 min to lyse the host strain.

5. The lysate was centrifuged for 10 min at 7000 rpm in a Sorvall (Model RC2-B) centrifuge with a SS-34 rotor (maximum radius 4.25 inches). The supernatant was collected and re-titered out on a plate, and the volume required for confluent lysis was noted.

6. Steps 1-5 were repeated for each clone.

(Clontech, 1994; Sambrook, et al., 1989)

5.2.8.3 Plate lysate preparation

For the preparation of large quantities of phage DNA there were two methods available. The liquid lysate method is more convenient than the plate lysate method, however phage generally grow better in plate lysates, so this method was chosen (Clontech, 1994).

1. For each selected clone, ten 15 cm petri dishes were plated out to confluent lysis, based on the titre of the high-titre stock of each clone. These plates were incubated overnight at 37°C.

2. 10 mL of 1X lambda dilution buffer was added to the ten plates and was left shaking at 4°C overnight.

3. The 1X lambda dilution buffer was pooled from the ten plates (~100 mL). This is the plate lysate.

(Clontech, 1994; Sambrook, et al., 1989)

5.2.8.4 Lysate processing

1. The lysate was placed into 50 mL Oakridge tubes and centrifuged at 8500 rpm on a Sorvall for 10 min at 4°C to pellet debris. A 10 mL aliquot of this lysate was kept as a very high titre stock.

2. The supernatant was decanted and pooled and DNase was added to 0.2 µg/mL and RNase to 5 µg/mL and left at room temperature for 15 min. This was to degrade the DNA and RNA from the host strain. The DNA of the EMBL3 SP6/T7 vector and insert was protected from the DNase by the phage's protein coat.

3. The lysate was separated again into Oakridge tubes and chloroform was added to a final concentration of 5% and vortexed for 30 sec. This was to lyse any cells that were not previously lysed.

4. The lysate was then centrifuged at 8500 rpm for 10 min at 4°C, to extract the organic components eg. lipid bilayer of cell membranes into the chloroform layer.

5. The aqueous supernatant was pooled and

- added solid NaCl to 1 M (eg. 100 mL/1000 mL x 58.44g = 5.84 g) and
- added polyethylene glycol (PEG) to 10% (eg. 10 g in 100 mL)
- The NaCl and PEG were dissolved and left on ice for at least one hour.

6. This was then centrifuged in Oakridge tubes at 8500 rpm for 15 min at 4°C. The supernatant was discarded. PEG and NaCl causes the phage to precipitate and form a whitish pellet on the bottom of the Oakridge tube. As much PEG solution was removed as possible, as it can inhibit restriction enzyme digests.

7. 2 mL of 1X lambda dilution buffer was added to each Oakridge tube, to dissolve phage pellet.

8. The phage was pooled into Oakridge tubes (~4 mL each) and CsCl was added to 0.5 g/m (ie. 2.0 g per Oakridge tube).

9. A CsCl step gradient was carefully layered in Beckman polyallomer centrifuge tubes (14 mm diameter x 89 mm). 2 mL of each CsCl concentration was added for the gradients (see Figure 5.3 & Table 5.1).

TABLE 5.1

CAESIUM CHLORIDE CONCENTRATION STEP GRADIENT

	Density (ρ)	CsCl (g)	1X LDB (mL)	Reference Index (n)
3	1.45	30.0	42.25	1.3768
2	1.50	33.5	41.0	1.3815
1	1.70	47.5	37.5	1.3990

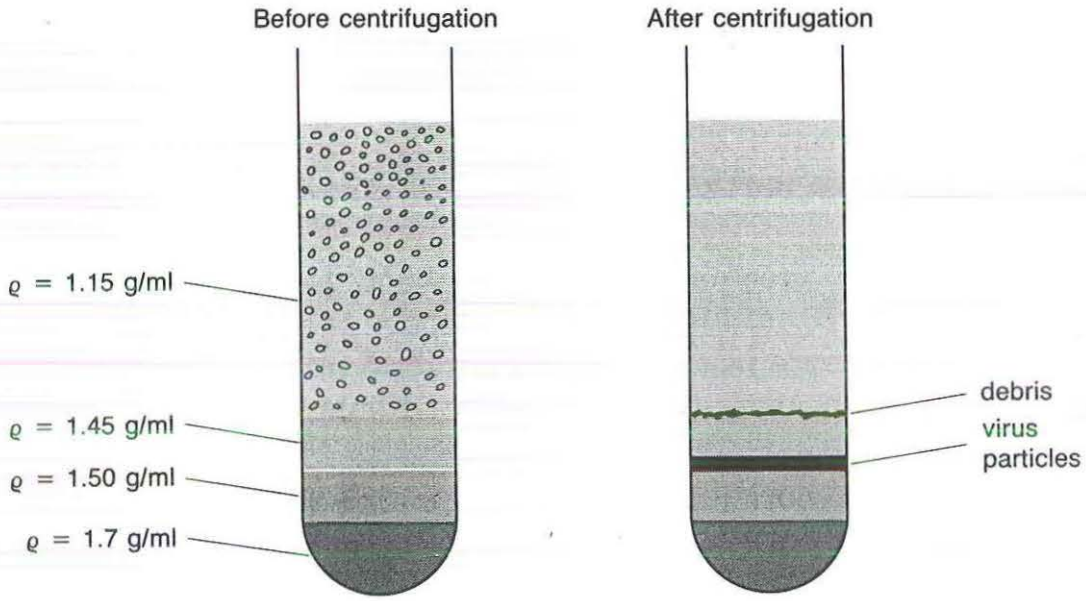
Solutions 1 to 3 were consecutively added, very carefully, to the Beckman tube, using a 5 mL pipette, so the gradient interfaces were well defined. The phage in 0.5 g/mL CsCl was added last.

FIGURE 5.3

Caesium Chloride Step Gradients to Purify Bacteriophage λ

The Bacteriophage λ forms a visible band at the 1.45 g/mL and 1.50 g/mL interface.

(After Sambrook et al., 1989)


Before centrifugation
After centrifugation
ϱ = 1.15 g/ml
ϱ = 1.45 g/ml
ϱ = 1.50 g/ml
ϱ = 1.7 g/ml
debris
virus particles

10. The opposite centrifuge capsules were weighed and balanced to a hundredth of a gram of each other and then centrifuged at 22 000 rpm, for 2 hrs, at 4°C, on a Beckman Model L8-M Ultracentrifuge.

11. The phage should have formed a visible band at the 1.45 g/mL and 1.50 g/mL interface. However, all debris between the 0.5 g/mL and 1.50 g/mL interface and the pellet on the bottom of the centrifuge tube was also collected and placed into different 1.5 mL eppendorf tubes. The different samples were collected using a 1 mL pipette with a new tip for each sample collected in the different gradients.

(ANRI, 1994; Clontech, 1994; Sambrook, et al., 1989)

5.2.8.5 DNA extraction

1. The collected volume from the 1.45 g/mL and 1.50 g/mL interface was pooled and 2X the volume of 1X lambda dilution buffer was added to dilute the CsCl.

2. The measured volume was made to:

20 mM EDTA

0.5% SDS

50 µg/mL Proteinase K

5 µg/mL RNase

This was incubated at 65°C for 15 min. The purpose was to digest any protein and RNA present.

3. Phenol/chloroform extraction. Equilibrated phenol:chloroform (1:1) was added to the same volume of phage DNA in an Oakridge tube and mixed together thoroughly for 5 min. This was then centrifuged at 15 000 rpm for 20 min on the Sorvall centrifuge. The chloroform denatures proteins and separates the protein into the organic phase and the nucleic acids into the aqueous phase (Sambrook, et al., 1989).

4. The supernatant was removed and Step 3 was repeated using the same volume of chloroform:isoamyl alcohol (24:1). The mixture was shaken thoroughly for 5 min and then centrifuged at 15 000 rpm for 20 min and the supernatant withdrawn. The isoamyl alcohol reduces foaming during extraction, caused by the SDS, to give a more defined interface (Sambrook, et al., 1989).

5. To the supernatant was added 2X volume of propan-2-ol. This was mixed thoroughly and centrifuged at 15 000 rpm for 10 min.

7. The DNA precipitated as a whitish smear on the bottom of the Oakridge tube. The supernatant was decanted and the DNA pellet was washed with 70% ethanol to remove added salts.

8. The DNA was resuspended in 1 mL of TE buffer.

9. The DNA concentration was measured on a spectrophotometer and the $A_{260/280}$ ratio should be between 1.8-2.0. The DNA was also run on a 0.8% agarose gel with a *Hind* III marker and should form a sharp band with some contaminating RNA.

(ANRI, 1994; Clontech, 1994; Sambrook, et al., 1989).

10. The DNA was purified by adding RNase to 5 µg/mL and using the Wizard™ DNA Clean-up System (Promega). The protocol was identical to the Wizard™ PCR Preps Purification System (Promega) in 2.2.6.2.

5.2.9 Restriction Digests of Clones to Determine if they are Different

The insert DNA can be excised with *Xho* I, *Sac* I, or *Sfi* I as can be seen from Figure 5.1. Other restriction enzymes were also tried including *Bam*H I, *Eco*R I and *Hind* III and different combinations of two enzymes, were also used. The purpose of the

restriction digests was to determine if the human inserts from the isolated clones were identical or different. The protocols used for the restriction digests were:

	1X 20 µL Reaction
Template (phage DNA)	empirically
10X Buffer appropriate to restriction enzyme (Promega)	2 µL
Acetylated BSA (1 mg/mL)	2 µL
Spermidine (0.1 M)	0.4 µL
Enzyme (Promega-usually 12 u/µL)	1 µL
H_2O to 20 µL	

All digests were at 37°C for one hour. Digests were terminated by heating to 95°C for 5 min.

5.2.10 Hybridising Radiolabelled Clones DNA to Genomic Blots

Approximately 1 µg of purified DNA from clones 2 and 4 were labelled by random primer labelling and hybridised to genomic blots as in 2.2.9. Clone 2 was hybridised to total human genomic DNA that had been cut with *Eco*R I. Clone 4 was hybridised to two blots on the same membrane of total human genomic DNA. One had been cut with *Bam*H I and the other with *Pvu* II.

5.2.11 Using Primers as Radiolabelled Probes on Membrane Lifts of the Colony Plaques to Determine if the Clones had the Same Part of the TPM2 Gene

5.2.11.1 Purpose for end-labelled primers experiment on clones

Primers DS 869.6F and 866.8skR were end-labelled radioactively and used as probes on the duplicate secondary membrane lifts of the four clones. This was to determine if the clones contained both exons 6 and 8sk, or if the clones were cut somewhere in between these exons. One primer was used as a probe on one of the secondary membrane duplicate lifts of the four clones. The other primer was used on the second lift. If one primer hybridised to the clone membranes at the previous positive plaque positions and the other primer didn't on the second lift, then it would confirm that the human insert was cut somewhere between the two primers in exon 6 and 8sk. If the two primers hybridised to the same plaque positions on both of the clones secondary membrane lifts, then it would confirm that the human inserts contained both exons 6 and 8sk.

5.2.11.2 Stripping membranes and prehybridisation using oligonucleotide hybridisation solution

The secondary membrane duplicate lifts (ie. 1A+B, 2A+B, 3A+B, 4A+B) were stripped of the previous hybridisation using the protocol in 2.2.9.8. The membranes were separated 1A-4A to hybridise with primer DS 866 (exon 8sk) and 1B-4B to hybridise with primer DS 869 (exon 6). The membranes were prehybridised in two separate plastic tubs using oligonucleotide prehybridisation solution.

Oligonucleotide prehybridisation solution is made up as for normal prehybridisation solution as in 2.2.9.5, except water is replaced for formamide. Formamide assists double stranded DNA to remain denatured or single stranded, which is not required with primers, as they are already single stranded.

10 mL/membrane or 40 mL of the oligonucleotide prehybridisation solution was added to each tub with the four membranes and incubated at 42°C, shaking in a hybridisation oven for 1 hour.

5.2.11.3 Kinase 5' end labelling of oligonucleotides

This procedure is useful for radioactive 5' end labelling of oligonucleotides (Promega, 1991). The kinase reaction requires that the 5' end has been previously dephosphorylated, and the primers are already synthesised with the 5' end dephosphorylated. Bacteriophage T4 polynucleotide kinase catalyses the transfer of the radiolabelled γ-phosphate of (γ-^{32}P)ATP (see Figure 5.4) to a 5' terminus of dephosphorylated DNA (Sambrook, et al., 1989).

Reaction:

$DNA_{OH}5'$ —(bacteriophage T4 polynucleotide kinase; (γ-^{32}P)ATP, dithiothreitol, Mg^{2+})→ 5'(^{32}P)DNA + ADP

Reaction:

	20 µL Reaction	Final Concentration
H_2O	4 µL	
Primers DS 866 or 869 (10 ng/µL)	6 µL	3 ng/µL
10X TM buffer (Tris-$MgCl_2$)	2 µL	50 mM Tris-HCl
(500 mM Tris-HCl, 100 mM $MgCl_2$)		10 mM $MgCl_2$
DDT (50 mM dithiothreitol)	2 µL	5 mM
(γ-^{32}P)ATP (10 µCi/µL)	5 µL	2.5 µCi/µL
T4 PNK (3u/µL)	1 µL	0.15 u/µL
Total	20 µL	

The reaction was incubated at 37°C for 30 min.

The reaction was stopped by heating to 94°C for 5 min, to denature the T4 enzyme.

The 5'-end radiolabelled primer DS 866 (exon 8sk) was added to the prehybridisation solution and membranes 1A-4A. The 5'-end radiolabelled primer DS 869 (exon 6)was added to membranes 1B-4B. The two hybridisations were left shaking overnight, at 42°C. The membranes were washed at increasing stringencies as in 2.2.9.7, until the activities were between 10-20 cpm. The membranes were autoradiographed with Dupont Cronex 4 X-ray film with Quanta III autoradiography intensifying screens at -80°C overnight, or until images were readable.

5.2.12 PCR Assay of DNA from Clones to Confirm that they Contained Part of the TPM2 Gene.

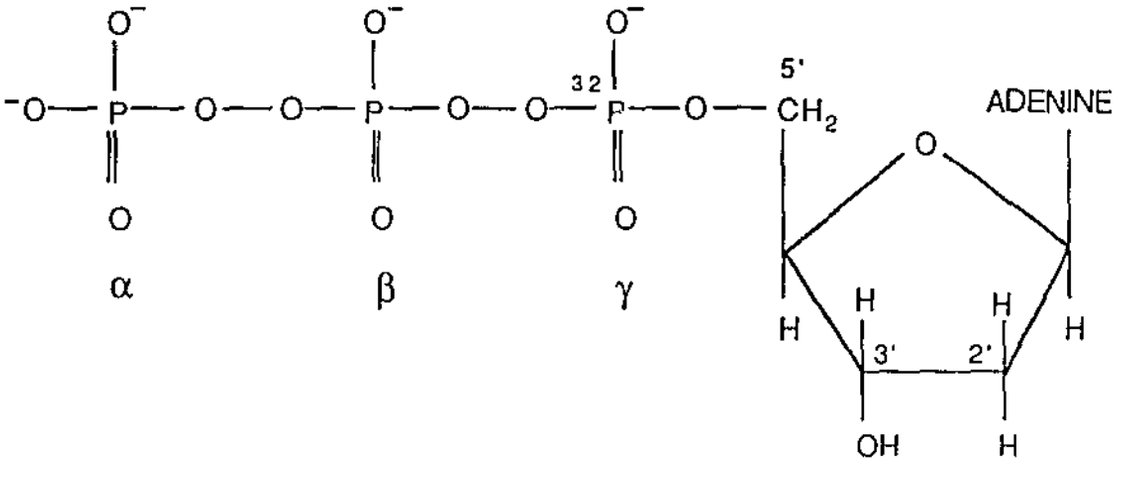
Primers DS 869 (exon 6) and DS 866 (exon 8sk) were used with the purified DNA from clones 2 and 4 to determine if the human inserts contained that part of the TPM2 gene. A PCR was set up with the following conditions:

For a 25 µL Reaction:-		**7X Reaction Mixture (µL)**
5X 2^{200} Buffer	5.0 µL	35
(5X Tth buffer, 10 mM $MgCl_2$, 1 mM dNTP's)		
Tth DNA polymerase (Biotech) (0.5 units/mL)	1.0 µL	7
Primer mix DS 866 + 869 (20 ng/µL)	2.0 µL	14
H_2O to 20 µL	12.0 µL	112
TOTAL	20.0 µL	
Paraffin 1 drop overlay		

FIGURE 5.4

(γ-^{32}P, ATP Showing the γ Phosphate Position

(After Wilton, 1993)


O⁻
O⁻
O⁻
⁻O
P
O
O
P
O
O
P
O
CH₂
5'
3 2
ADENINE
O
O
O
α
β
γ
H
H
H
H
3'
2'
OH
H

Target DNA

2X clone 2 DNA diluted 1/100X (~10 ng/μL)	1 μL and 4 μL of H_2O
2X clone 4 DNA " " " "	1 μL and 4 μL of H_2O
1X Human Genomic DNA as positive control (10 ng/μL)	5 μL
1X Blank (H_2O)	5 μL

20 μL of the reaction mixture was added to the target DNA.

PCR Cycling Conditions: 2 Step on Automatic Thermal Cycler

		time	
temp	94°C	5 min	30 sec (denaturing)
	58°C		6 min (annealing and extension)
# cycles		x1	x35
	4°C		indefinitely

5.2.13 Sequencing the Clones and PCR Assays to Confirm they were Specific to TPM2

Purified DNA from clones 2 and 4 was sequenced directly to confirm that they contained part of the TPM2 gene. Because the human insert is only a portion of the phage DNA more template DNA is required for sequencing. ABI protocols suggest using 1 μg of phage DNA when sequencing (Applied-Biosystems, 1993). The protocols were the same as 2.2.8.

The 1.5 kb TPM2 STS assay (using clones 2 and 4 DNA as the template from 5.2.12) was also sequenced to confirm the clone's inserts were specific to TPM2. 200-300 ng of TPM2 STS was used as the template for the sequencing as in 2.2.8.

5.3 RESULTS AND DISCUSSION

5.3.1 Determining if the 1.5 kb TPM2 STS was in the Genomic Library

The 1.5 kb TPM2 STS primers were used on the EMBL3 SP6/T7 library to determine if there was a clone that contained that part of the TPM2 gene (exon 6-8sk). In Figure 5.5, lanes 1-3 show the STS product from the EMBL3 SP6/T7 genomic library. Lanes 4-6 show the STS using total human genomic DNA as a positive control. Therefore, there were clone(s) present in the genomic library that contained exon 6-8sk of the gene and the library could be used to isolate clones containing that part of the TPM2 gene for use in FISH.

The DNA from the λ phage was able to be targeted by the primers, because the initial heating period of 5 min denatured the phage's protein coat.

5.3.2 Primary and Secondary Screening of the Plaque Membrane Lifts

The initial titering of the eight plates to represent one genomes worth of DNA produced an average of 50 000 pfu/plate, as was calculated. The plates were near confluent and were ideal for the first screening of the duplicate membrane lifts. The labelling efficiency of the 1.5 kb TPM2 STS was approximately 50%, as estimated by the PEI strip after the random priming reaction.

Out of the eight plates that were membrane lifted, there were four duplicate plaques that were positive on the autorads (See Figure 5.6). The agar plugs contained several plaque colonies and not just the one that was positive, because the titre was so high and the plaques were so close to each other. These four plaque colonies were picked as agar plugs and replated for a secondary screening (see Figure 5.7).

FIGURE 5.5

1.5 kb TPM2 STS (Exons 6-8sk) PCR Assay of the Genomic Library.

Lanes 1-3	is the STS Present in the Genomic Library.
Lanes 4-6	is the STS from Human Genomic DNA.
Lane 7	Blank
Lane 8	λ Pst I Marker

2% Agarose Gel, 100V for 45 min

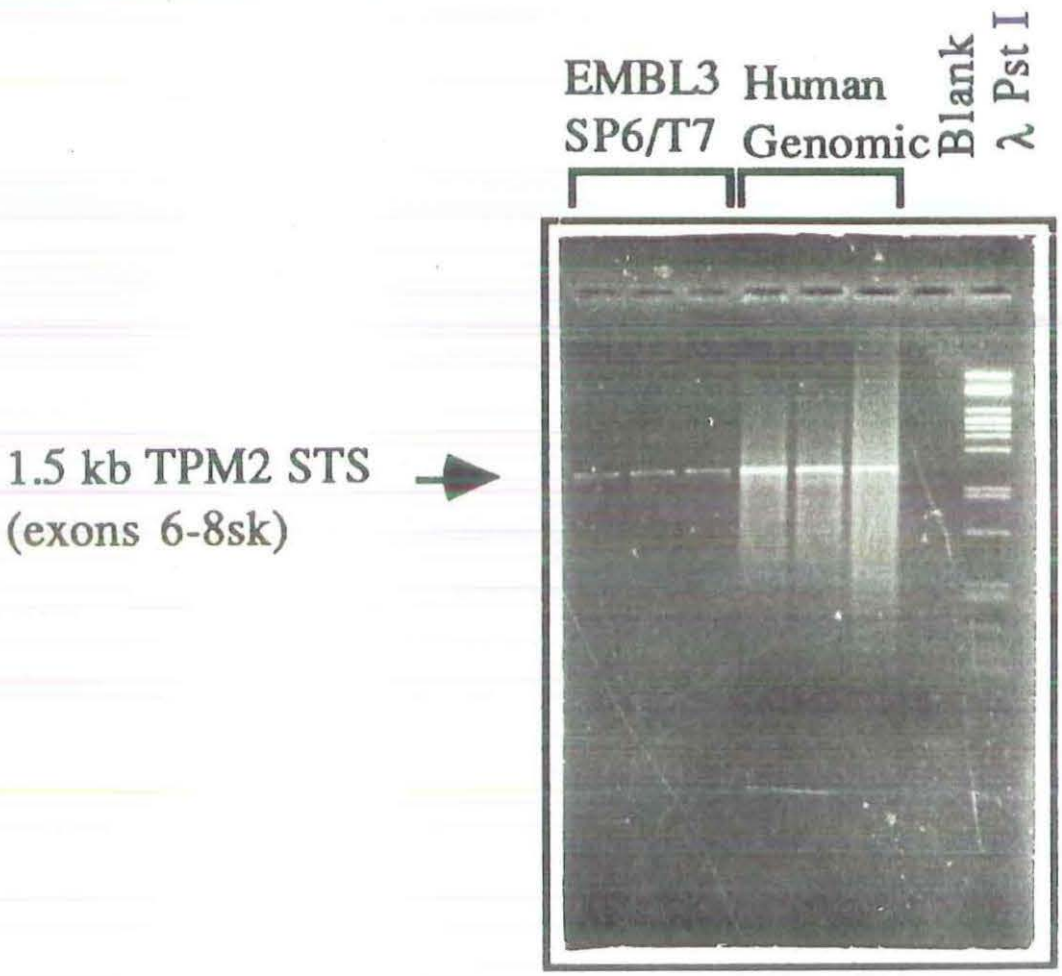
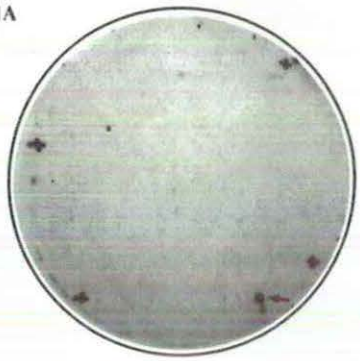

EMBL3
SP6/T7
Human
Genomic
Blank
λ Pst I
1.5 kb TPM2 STS
(exons 6-8sk)

FIGURE 5.6

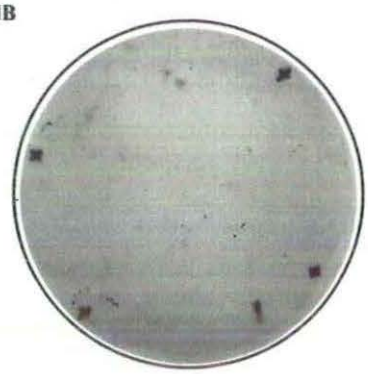
Primary Screening Of Duplicate Membrane Plaque Lifts With Radiolabelled 1.5 kb TPM2 STS (Exons 6-8sk). Those Plaques that showed up in Duplicate were Considered Positive for Containing that Part of TPM2. The Arrows Identify the Clones that were picked for a Secondary Screening. Developed -80°C, for 16 hours with 1 intensifying screen.

DUPLICATE MEMBRANE LIFTS

1A

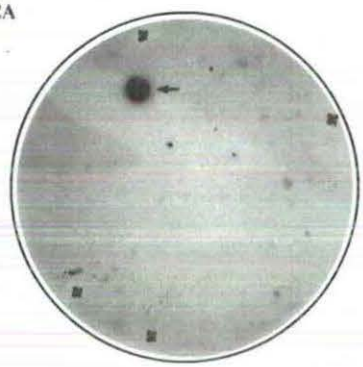


1B



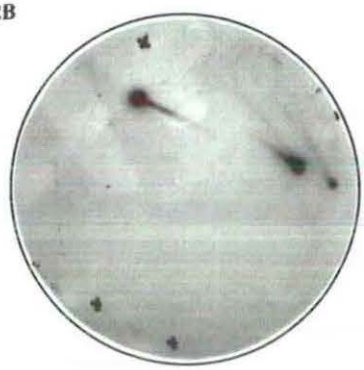
CLONE 1

2A

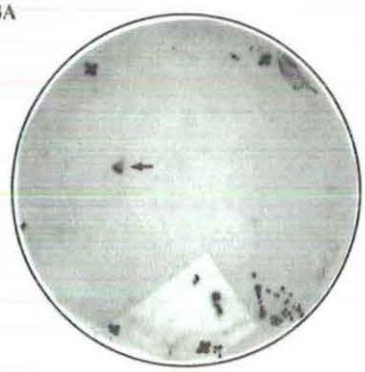


CLONE 2

2B

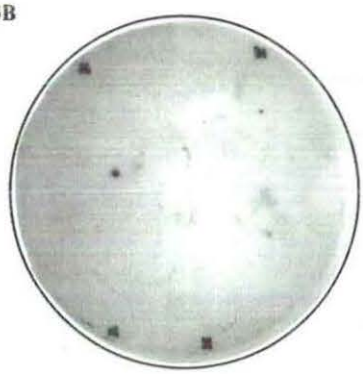


3A

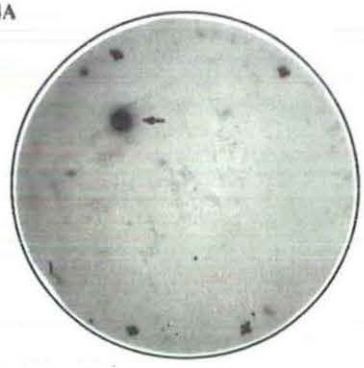


CLONE 3

3B



4A



CLONE 4

4B

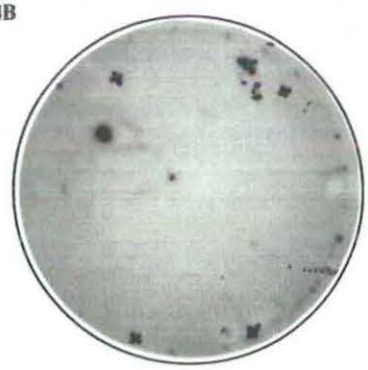
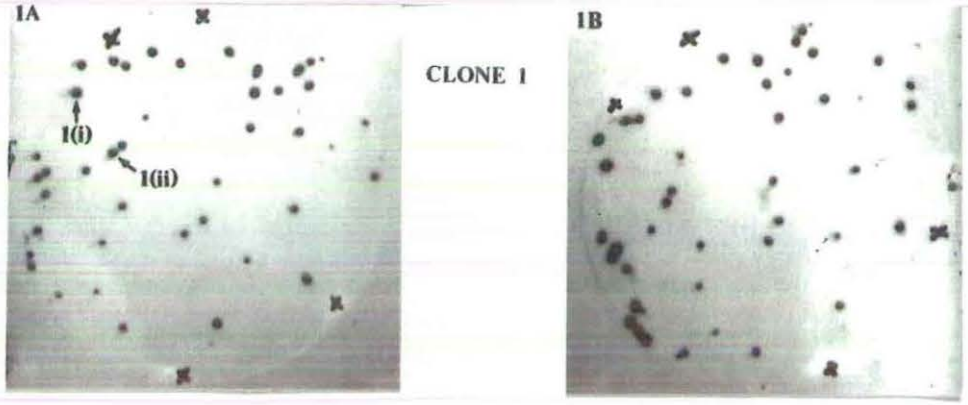
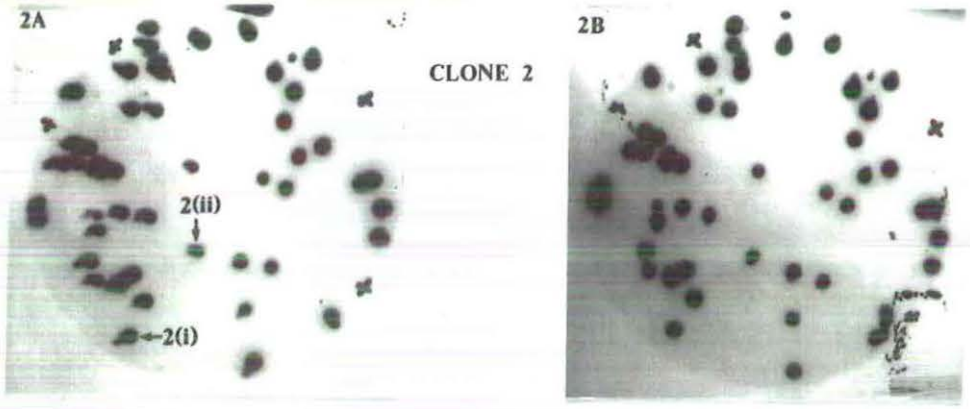


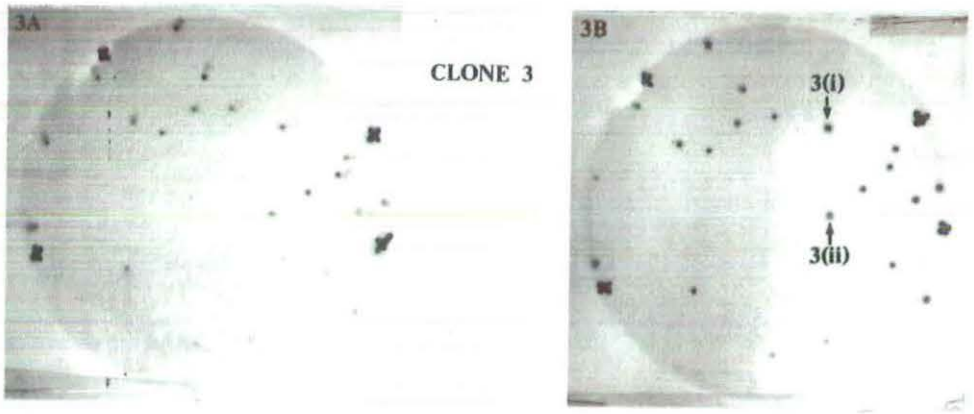
FIGURE 5.7

Secondary Screening of Duplicate Membrane Plaque Lifts.
Those Plaques that showed up in Duplicate were
Considered Positive for Containing that Part of TPM2.
The Labelled Arrows Represent the Clones that were picked
for Tertiary Screening.
Developed -80°C, for 18 hours with 1 intensifying screen.

SECONDARY DUPLICATE MEMBRANE PLAQUE LIFTS







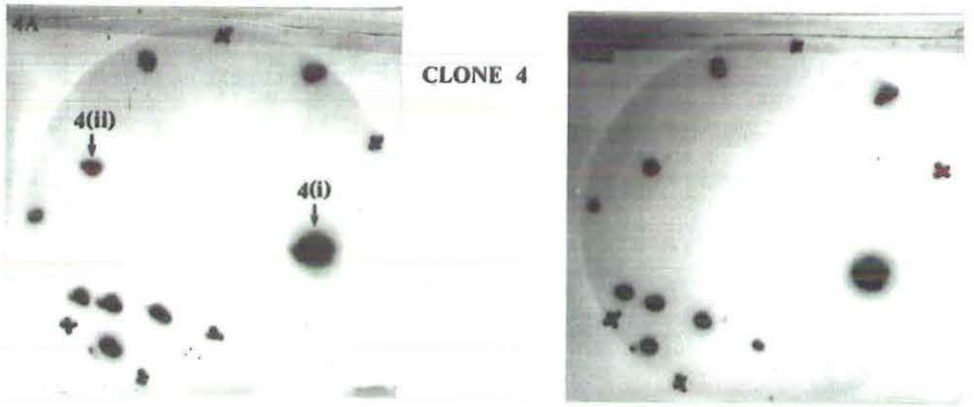
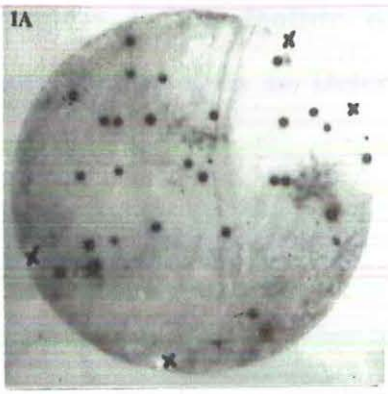


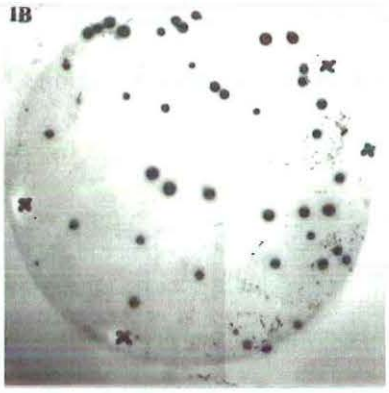
FIGURE 5.8

5' End Labelling of Primers DS 869.6F & DS 866.8skR on the Secondary Membrane Lifts. Compare the Plaque Positions with Figure 5.7. Developed -80°C, for 24 hours with 1 intensifying screen.

SECONDARY MEMBRANE LIFTS PROBED WITH TPM2 PRIMERS

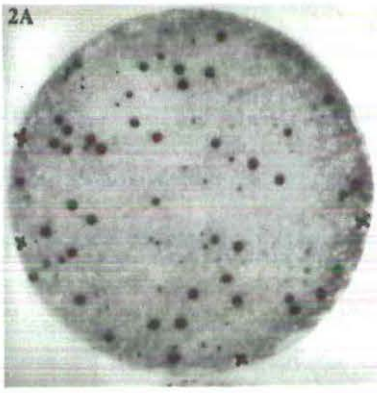


CLONE 1

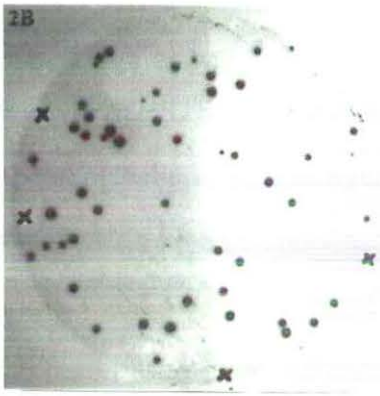


PRIMER DS 866 EXON 8sk

PRIMER DS 869 EXON 6



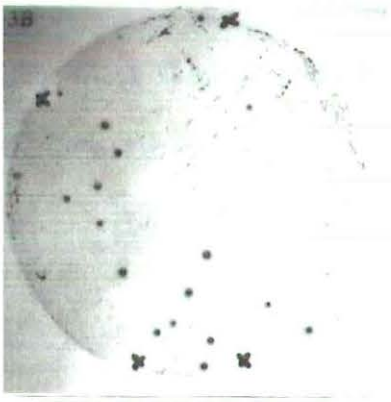
CLONE 2



PRIMER DS 866 EXON 8sk

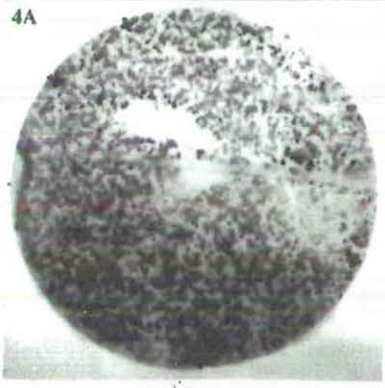
PRIMER DS 869 EXON 6

CLONE 3

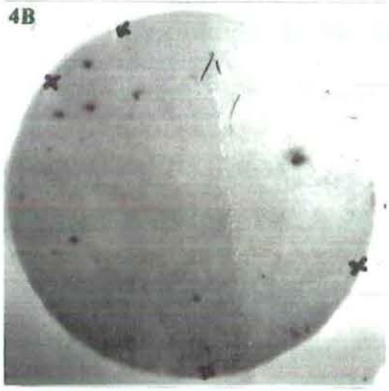


PRIMER DS 866 EXON 8sk

PRIMER DS 869 EXON 6



CLONE 4



PRIMER DS 866 EXON 8sk

PRIMER DS 869 EXON 6

5.3.3 Primers Polynucleotide Kinase 5' End-Labelled as Probes on the Secondary Plaque Lifts to Determine if the Clones Contain both Exons 6 and 8sk

It was noted two of the (clones 2 & 4) had stronger positive signals on the autorads than the other two clones (1 & 3). When the secondary screening was done this pattern was repeated, clones 2 and 4 gave much stronger positive signals than clones 1 and 3 on the autorad (Figures 5.6 & 5.7).

It was suspected only part of the radiolabelled 1.5 kb TPM2 STS was hybridising to clones 1 and 3 giving the weak signal, while all of the radiolabelled STS was hybridising to clones 2 and 4 giving stronger signals. The radiolabelled 1.5 kb STS contained exons 6 to exon 8sk. It was therefore hypothesised that clones 1 and 3 only contained part of this STS. The primers DS 869 (exon 6) and DS 866 (exon 8sk), were polynucleotide kinase (PNK) 5' end labelled and one probe each was hybridised on one of the duplicate secondary membrane lifts. If one primer hybridised to clones 1 and 3 membranes at the previous positive plaque positions and the other primer didn't on the second lift, then it would confirm that the human insert was cut somewhere between the two primers in exon 6 and 8sk. If the two primers hybridised to the same plaque positions on clones 2 and 4 secondary membrane lifts, then it would confirm that the human inserts contained both exons 6 and 8sk.

In Figure 5.8, for clones 2 and 4, the positive plaque positions were identical on the duplicate autorads and both primers hybridised to the same plaques as the original secondary screening (compare Figures 5.8 and 5.7). Therefore, clones 2 and 4 both contained exon 6 and 8sk in the human insert.

However, for clones 1 and 3 the duplicate positive plaques were not identical and only the labelled primer DS 869.6F (exon 6) hybridised to the same plaques as the original secondary screening. The other primer DS 866.8skR (exon 8sk) for clones 1 and 3

hybridised to non-matching plaques from the original secondary screening (compare Figures 5.7 and 5.8). This meant that the human insert for clones 1 and 3 did not contain exon 8 and that the insert was cut somewhere between the 1.5 kb between exons 6 and 8sk.

5.3.4 PCR Assay on Clones 1-4 Eluates

To confirm that clones 1 and 3 inserts were cut between exons 6-8sk in TPM2, a PCR assay using primers DS 869.6F and 866.8skR was performed on eluates from plaque colonies that had been selected. This was compared with the PCR assay of clones 2 and 4 eluates, which from the PNK experiment showed that these clones contained exons 6-8sk. 5 µL of eluted phage from the clone eluates was used as template DNA for the primers.

From Figure 5.9, it can be seen that the primers did not amplify the 1.5 kb TPM2 STS from clone 1 (lanes 1-4) and clone 3 (lanes 7-11) insert DNA, but the STS was amplified on clone 2 (lanes 5 & 6) and clone 4 (lanes 12-13) eluates. This confirmed the PNK experiment that clones 1 and 3 insert DNA was cut somewhere between exons 6-8sk in the TPM2 gene and clones 2 and 4 contained these exons.

Therefore, from the positive results, clones 2 and 4 were chosen for isolation of DNA using the plate lysate method as in 5.2.8. This was after the clones 2 and 4 were rescreened five times so that almost 100% of the plaques showed up as positive.

FIGURE 5.9

PCR Assay of Clone Eluates

Lanes 1-4	Clone 1 different chosen plaque eluates.
Lanes 5-6	Clone 2 " " " "
Lanes 7-11	Clone 3 " " " "
Lanes 12-13	Clone 4 " " " "
Lane 14	Human Genomic DNA (Positive Control)
Lane 15	λ *Pst* I Marker

2% Agarose Gel, 100V for 30 min

FIGURE 5.10

Clones 2 & 4 DNA on a 0.8% Agarose Gel

Lane 1	Clone 2 Lambda DNA and Human Insert (Before Purification)
Lane 2	Clone 2 " " " " " (After Purification)
Lane 3	Clone 4 " " " " " (Before Purification)
Lane 4	Clone 4 " " " " " (After Purification)

0.8% Agarose Gel, 100V for 1 hr

~45 kb
Clone 2 Unpurified
Clone 2 Purified
Clone 4 Unpurified
Clone 4 Purified
λ Pst I

1.5 kb TPM2 STS
(exons 6-8sk)
Clone 1
Clone 2
Clone 3
Clone 4
Genomic DNA
λ Pst I

5.3.5 Lysate Processing

The phage lysate from clones 2 and 4 were processed as in 5.2.8.4. The DNA from the caesium chloride gradient did not form a distinct band at the 1.45 g/mL and 1.50 g/ml interface. There was a debris band in the 1.45 g/mL gradient and a faint band below this but the gradient interfaces were difficult to see after centrifuging. A pellet also formed on the bottom of the centrifuge tube. This made it difficult to collect the DNA, but the faint band and the pellet at the bottom were collected and placed into different eppendorf tubes. In future, a better technique would be to add to the phage 0.5 mg/mL of $CsCl_2$ in 1X lambda dilution buffer, with 0.5 µg/mL ethidium bromide. After centrifuging the tube could be placed over a transilluminator and the DNA extracted where it fluoresces in the tube.

5.3.6 DNA Extraction

The extracted phage DNA in 5.2.8.5 from clones 2 and 4 was electrophoresed on a 0.8% agarose gel at 100V for one hour (see Figure 5.10). To the phage DNA was added RNase (5 µg/mL) and proteinase K (50 µg/mL) and incubated at 65°C for 15 min, to digest any RNA and protein present. The DNA was run on a low melting point agarose and excised and purified using the Wizard DNA Cleanup System (Promega). It can be seen the phage DNA before purification was contaminated with RNA by the white smear at the bottom of lanes 1 & 3, which was not present after purification (lanes 2 & 4). Because RNA has a similar absorbance at 260 nm (RNA 1A=40 ng/µL) to DNA (DNA 1A=50 ng/µL), accounts for the very high nucleic acid concentration of the unpurified DNA as seen in Table 5.1. The recovery efficiency of DNA after purification from the gel was quite low, using the Wizard DNA Clean-up System (Promega) as can be seen in Figure 5.10 from the intensity of the bands before and after purification.

Table 5.2

EMBL3 Clone's DNA Concentrations on Spectrophotometer

	Mean A_{260}	Mean A_{280}	A_{260}/A_{280}	Dilution	Concentration (ng/μL)
Clone 2 (Unpurified)	0.73	0.39	1.88	200X	7 300
Clone 2 (Purified)	0.039	0.019	2.1	25X	50
Clone 4 (Unpurified)	0.312	0.169	1.84	700X	10 000
Clone 4 (Purified)	0.012	0.007	1.6	25X	15

Approximately 1 mL of clones 2 and 4 were obtained from the DNA extraction in 5.2.8.4-5. From the spectrophotometer readings, 50 μg (50 ng/μL = 50 μg/mL) of clone's 2 DNA and 15 μg of clone's 4 DNA was isolated. This compared well the expected recovery of 20 - 40 μg of phage DNA, from the approximate 100 mL volume of lysate (Clontech, 1994).

5.3.7 Restriction Digests of Clones 2 and 4 DNA to Determine if they were the same Clones

The restriction enzymes used on clones 2 and 4 DNA were *Bam*H I, *Eco*R I and *Hind* III. Each clone's DNA was cut with the different enzymes and all the combinations using two enzymes per digest. This was to determine if the clones were identical, or if the human inserts were different. Figure 5.11 shows the different combinations of restriction digests on clones 2 and 4, run on a 1% agarose gel, at 50V for 4 hours. It can be seen clearly that the two clones are different each having different human inserts. For example, in lanes 3 and 4 (clones 2 and 4 cut with BamH I) there are three distinct bands in lane 4, clone 4, that are not present in lane 3, clone 2.

FIGURE 5.11

Restriction Digest of Clones 2 and 4 DNA

Lane 1	λ *Hind* III Marker
Lane 2	λ *Pst* I Marker
Lane 3	Clone 2 cut with *Bam*H I
Lane 4	Clone 4 " " "
Lane 5	Clone 2 cut with *Eco*R I
Lane 6	Clone 4 " " "
Lane 7	Clone 2 cut with *Hind* III
Lane 8	Clone 4 " " "
Lane 9	λ *Hind* III Marker
Lane 10	λ *Pst* I Marker
Lane 11	Clone 2 cut with *Bam*H I and *Eco*R I
Lane 12	Clone 4 " " " " "
Lane 13	Clone 2 cut with *Bam*H I and *Hind* III
Lane 14	Clone 4 " " " " "
Lane 15	Clone 2 cut with *Eco*R I and *Hind* III
Lane 16	Clone 4 " " " " "
Lane 17	λ *Hind* III Marker
Lane 18	λ *Pst* I Marker
Lane 19	Clone 2 Uncut
Lane 20	Clone 4 Uncut

The products of the digests were run on a 1% agarose gel, at 50V for 4 hours.

FIGURE 5.12

1.5 kb TPM2 STS of Clones 2 And 4 Purified DNA.

2% Agarose Gel, 100V for 45 min

1.5 kb TPM2 STS
(exons 6-8sk)
λ Pst I
Clone 2
Clone 4

λ Hind III
λ Pst I
2 4 2 4 2 4 λ
BamH I
EcoR I
Hind III
Hind III
Pst I
2 4 2 4 2 4 λ
BamH I & EcoR I
BamH I & Hind III
EcoR I & Hind III
Hind III
Pst I
2 4 λ
Uncut

From Figure 5.1 showing the restriction map of EMBL3 SP6/T7, and Figure 2.12 showing the restriction map of the 1.5 kb TPM2 STS, it can be seen none of the restriction enzymes cut either the vector arms or the exon sequence of the TPM2 STS. Therefore any lanes that showed more than two bands was due to the restriction enzymes cutting the insert DNA, other than the exon sequence (exons 6-8sk) of the STS.

This meant two different clones that contained part of the TPM2 gene had been isolated and could be used in FISH to verify each others localised position.

The size of clones 2 and 4 inserts cannot be determined precisely from Figure 5.10, but can be estimated from the restriction digests in Figure 5.11. It was assumed all the lower bands, were the insert DNA, because none of the enzymes used cut the vector EMBL3 SP6/T7 arms, as can be seen in Figure 5.1. Therefore, the lower molecular weight bands must have been insert DNA that was cut. Using the λ *Hind* III and λ *Pst* I markers, the band sizes can be added to give an approximate insert size.

For clone 4 in lane 12, three lower bands can be seen (1.5, 2 and 3.5 kb) that add up to 7 kb and if the band above these is 8 kb, then this gives an approximate insert size of 15 kb.

For clone 2 on another digest (data not shown) the insert was slightly larger than clone 4 and was estimated to be approximately 16-18 kb.

5.3.8 PCR Assay of Clones DNA Using the 1.5 kb TPM2 STS

Primers DS 869 (exon 6) and DS 866 (exon 8sk) were used on the purified clones 2 and 4 DNA to determine if the human inserts contained the 1.5 kb TPM2 STS. Figure 5.12 shows the STS from clones 2 and 4 in lanes 2 and 3 respectively with the λ *Pst* I marker in lane 1. Human genomic DNA was used as a positive control in lane 4, but it did not

amplify. This showed that the purified DNA from clones 2 and 4 contained the 1.5 kb TPM2 STS from exons 6 to 8sk.

5.3.9 Sequencing Clones 2 and 4 DNA and the 1.5 kb TPM2 STS

The most direct way of confirming that clones 2 and 4 contained part of the TPM2 gene was to sequence the insert DNA using primers DS 869.6F (exon 6 forward) and DS 866.8R (exon 8sk reverse). 1 µg of each clone was used to allow ensure sufficient template. However, no useable sequence was obtained after using this method a few times.

The next most direct method, was to sequence the 1.5 kb TPM2 STS using the two clones as template DNA. Figure 5.13 shows the sequence of the TPM2 STS from clone 4 which has been summarised into Table 5.2. The sequence extended for approximately 450 bases from primer DS 869 in exon 6, to intron 6, exon 7 and into intron 7. When the sequencing results were compared with that of Widada et al. (1988) TPM2 sequence, out of 70 bases in exon 7 only one base was different and two bases could not be read (ie. N). The one base that was different in exon 7 was a thymine from Widada's sequence was substituted for an adenine. The thymine is conserved in all the TPM's. The base mismatch is not likely to be real because the adenine was next to a 'N' in the sequence, so it was not sequencing well at that point. The sequence variations were likely to be due to the template preparation, the sequencing reaction or the sequencer tracking, reading and analysing the fluorescent end labelled products. The sequencing from the reverse primer (DS 866.8skR) in exon 8sk was not useable.

FIGURE 5.13

Sequence of TPM2 STS using Clone 4 as Template DNA from Primer DS 869.6F in Exon 6.

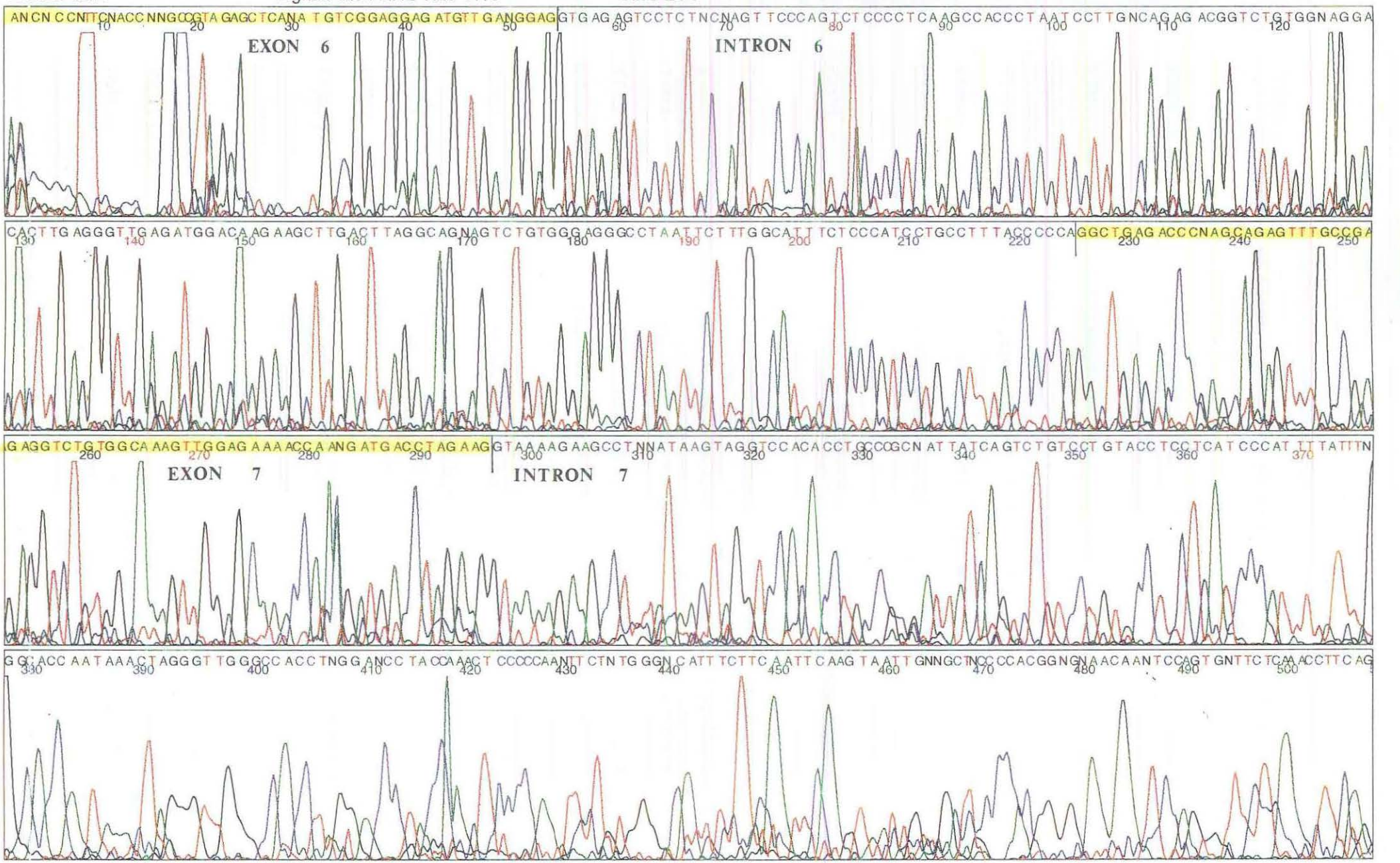

EXON 6
INTRON 6
EXON 7
INTRON 7

Table 5.3

Sequence of Clone 4 compared to TPM2 and other TPM's

EXON 6

TPM1	tcctttccga caagctgaag gag
TPM3	ttcttactga taaactcaag gag
TPM4	ttctgtctga caaactgaaa gag
TPM2	tgttggagga gaagctgaag gag
Clone 4	tgtcggagga gatgttgang gag

EXON 7

TPM1	gctgagactc gggctgagtt tgcggagagg tcagtaacta
TPM3	gcagagaccc gtgctgagtt tgctgagaga tcggtagcca
TPM4	gctgagaccc gtgctgaatt tgcagagaga acggttgcaa
TPM2	gctgagaccc gagcagagtt tgccgagagg tctgtggcaa
Clone 4	gctgagaccc nagcagagtt tgccgagagg tctgtggcaa

TPM1	aattggagaa aagcattgat gacttagaag
TPM3	agctggaaaa gacaattgat gacctggaag
TPM4	aactggaaaa gacaattgat gacctggaag
TPM2	agttggagaa aaccatcgat gacctagaag
Clone 4	agttggagaa aaccaangat gacctagaag

From the TPM2 STS sequence of exon 7, it matched Widada et al. published sequence by 96%. The underlined bases for TPMs 1, 3 and 4 and clone 4 are those bases that differ to the TPM2 sequence. The nine bolded bases in clone 4 are those bases that are only unique to TPM2. Therefore, it can be confident that clone 4 was specific to TPM2.

Anthony Akkari at ANRI, also sequenced the STS product from primer DS 869.6F and out of 35 readable bases in exon 6 only one base could not be read (ie. N) and all 70 bases in exon 7 matched Widada et al. sequence and also confirmed that clone 4 was specific to TPM2.

Clone 2's sequencing results were not useable, but from the 1.5 kb TPM2 STS that was amplified from the clone's DNA as template, it can be confident that the clone 2 was also specific to TPM2.

Anthony Akkari also sequenced clone 2 from the reverse primer DS 866.8skR. Out of 54 readable bases in exon 8sk, only three bases could not be read (ie. N) and matched Widada et al.'s sequence by 94% and therefore confirmed that clone 2 was specific to TPM2 .

5.3.10 Hybridising Clones DNA to Genomic Blot

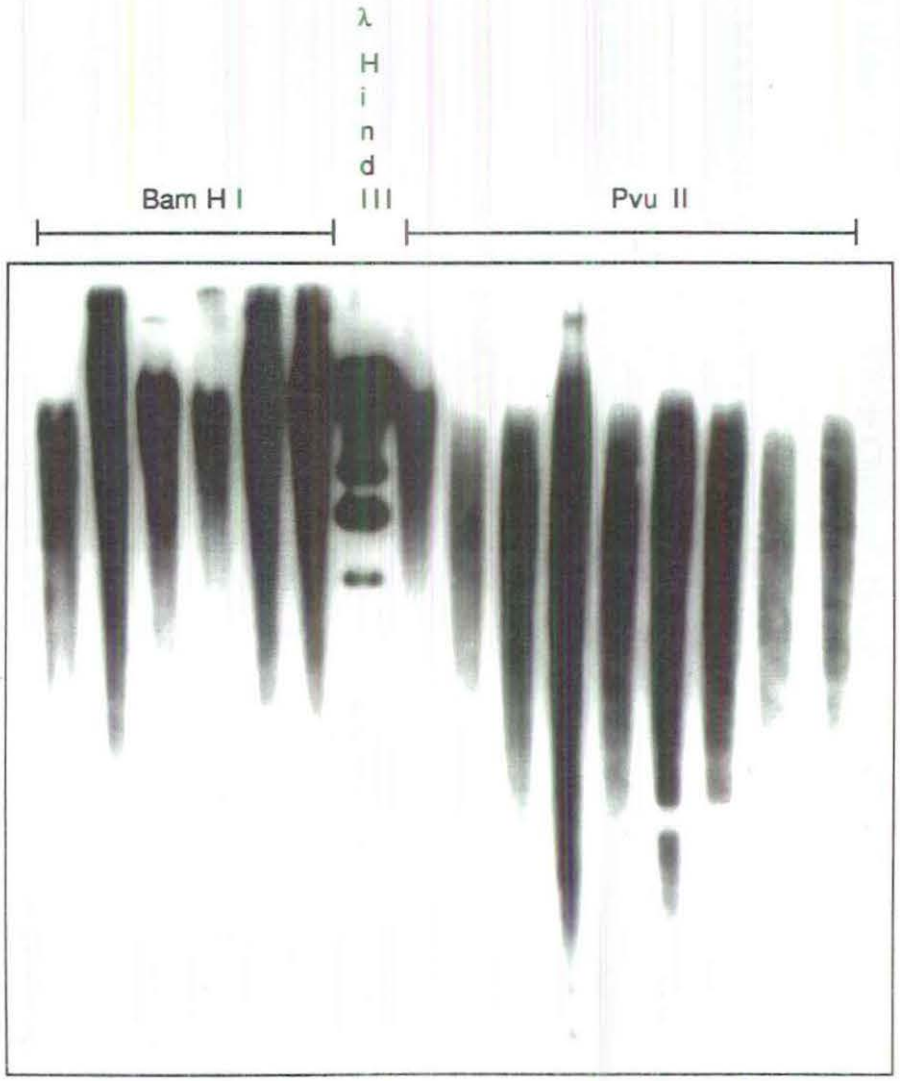
1 μg of clone 4 DNA was radiolabelled and hybridised to a human genomic blot that had been cut with *Bam*H I and *Pvu* II. In Figure 5.14, the human DNA in lanes 1-6 and 8 and had been cut with *Bam*H I. Lane 7 was a λ *Hind* III marker and lanes 9-16 were human DNA that had been cut with *Pvu* II. There were no discrete consistent bands across either digest, except in lane 7 where the EMBL3 SP6/T7 arms, hybridised with the λ phage DNA that had been cut with *Hind* III. The probe hybridised unspecifically to the digested human DNA as there were only smears in the lanes. It was hoped that the probe would hybridise at distinct bands, indicating that the probe insert would hybridise well with genomic DNA, especially when used for FISH.

Clone 2's hybridisation to human DNA that had been digested with *Eco*R I was also unsuccessful as not enough template DNA was labelled (data not shown).

The experiment was not repeated as the use of the labelled clones on genomic blots was not essential to the project. It only gives an indication of the hybridising ability of the clones to genomic DNA and time spent optimising the conditions was not a priority. The clones may still hybridise successfully in FISH.

FIGURE 5.14

Radiolabelled Clone 4 DNA Hybridised to a Genomic Blot


λ
H
i
n
d
III
Bam H I
Pvu II

CHAPTER 6

USE OF GENOMIC CLONES FOR FISH

6.1 INTRODUCTION

Approximately 10 μg of clones 2 and 4 DNA was sent to the Department of Cytogenetics and Molecular Genetics, at Adelaide Children's Hospital, for use in FISH.

6.2 MATERIALS AND METHODS

The same methods were used as in 4.2.

6.3 RESULTS AND DISCUSSION

Clones 2 and 4 were used for FISH to metaphase spreads from three normal males. Twenty-five metaphases from the first normal male were examined for fluorescent signal with clone 2. Eighteen of these metaphases showed signal on one or both chromatids of chromosome 9 in the region 9p13-p21; 95% of this signal was at 9p13.1 (see Figure 6.1). There was a total of 11 non-specific background dots observed in these 25 metaphases. A similar result was obtained from hybridisation of this probe to 15 metaphases from the second normal male and from hybridisation using the probe of clone 4 to 20 metaphases from the 3rd normal male (data not shown) (Hunt, et al., 1995).

The 1.5 kb TPM2 STS that was used as a probe for FISH, didn't work, yet the genomic clones 2 and 4 were both successful in FISH. All probes were reduced to 200-500 bp fragments by the nick translation, and so were likely to be equally specific as probes for the TPM2 gene. It was likely the greater amount of labelled DNA in the genomic clones (~15-18 kb) was able to hybridise to the metaphase chromosomes and give a stronger

signal, than the 1.5 kb TPM2 STS. This probably explains why the genomic clones worked in FISH and the 1.5 kb TPM2 STS was unsuccessful.

The localisation to 9p13.1 confirmed and refined the somatic cell hybrid panel localisation to chromosome 9.

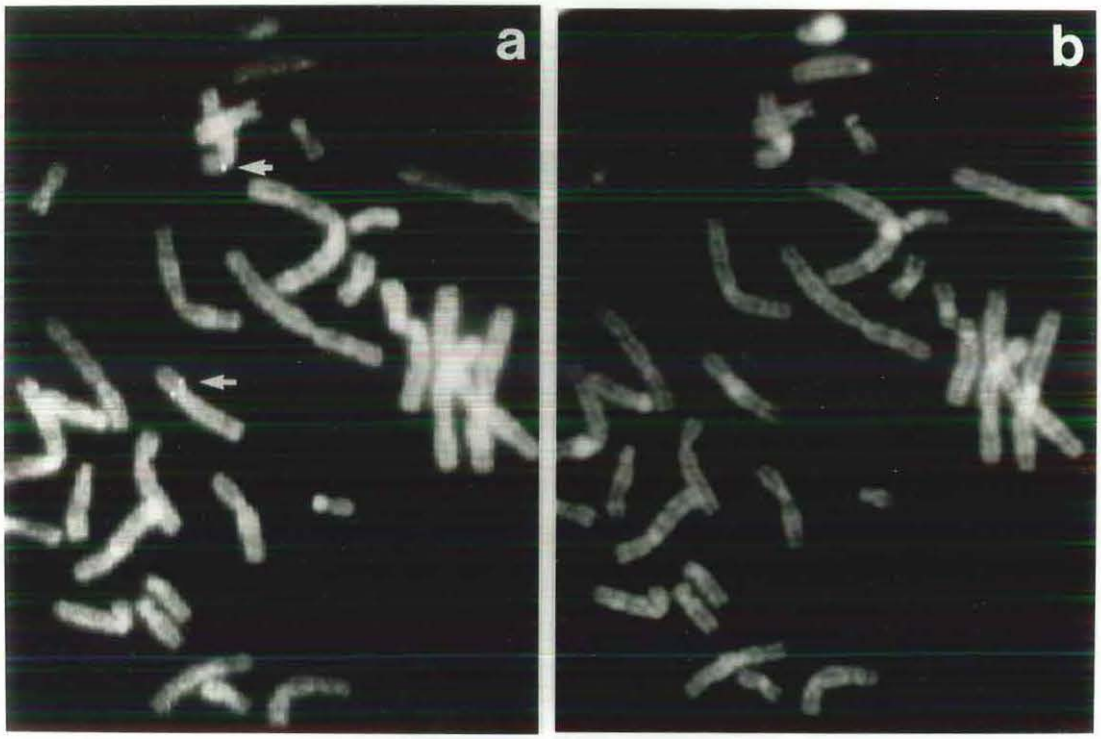
FIGURE 6.1

Metaphase showing FISH with the probe TPM2.2.

(a) Normal male chromosomes stained with propidium iodide. Hybridisation sites on chromosome 9 are indicated by arrows.

(b) The same metaphase as (a) stained with DAPI for chromosome identification.

TPM2 FISH TO 9p13.1



CHAPTER 7

IDENTIFICATION OF A POLYMORPHISM IN THE TPM2 GENE

7.1 INTRODUCTION

The majority of human DNA is not involved in coding sequences and is therefore not subject to tight selection. If the sequence is compared in a noncoding autosomal region from 10 individuals, then a nucleotide will be found approximately every 200 bp at which there is a sequence variation (Gelehrter & Collins, 1990). These variations are usually due to simple base changes and are called DNA polymorphisms (Gelehrter & Collins, 1990). A DNA polymorphism is, then, is a region of variable sequence between different individuals.

A method to find polymorphisms involves testing approximately 10 people. If one allele is polymorphic then the frequency of alleles having the polymorphism is approximately 5%, as 10 people each have two alleles. A polymorphism with a frequency less than this would not be generally useful as a marker in the genome, as very few families would be informative in demonstrating Mendelian segregation. If a polymorphism is identified, then its frequency can be refined by screening 50 - 100 individuals (Cichon, et al., 1994; Rolfs, et al., 1994; Shen, et al., 1994).

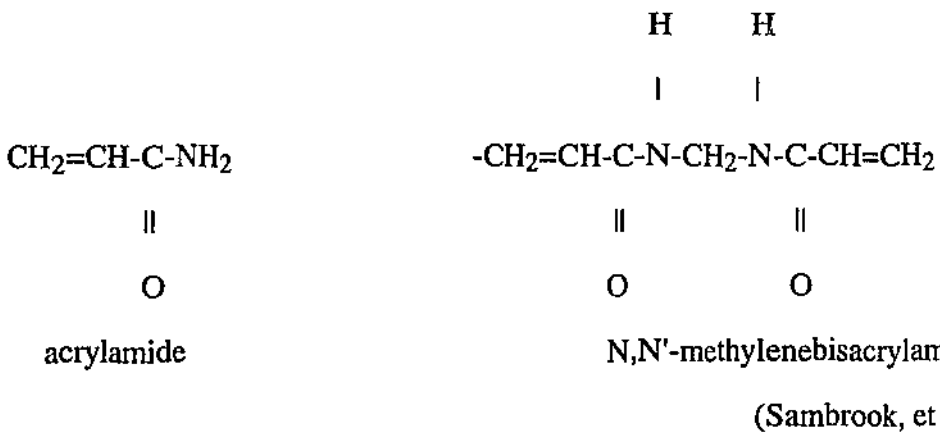
A technique to identify sequence variation is the single stranded conformation polymorphisms (SSCP). SSCP is where a PCR amplified segment of the gene (100-350 bases) is denatured into single strands and snap chilled so the fragments collapse in the most stable single stranded tertiary form. The PCR incorporates a radioactive nucleotide such as (α-P^{32}) dCTP. The strands are separated on a nondenaturing polyacrylamide gel under constant voltage so the polymorphisms keep their tertiary forms. Any fractionated bands that are different for different individuals indicate a polymorphism. These can be

cut out, reamplified by PCR and sequenced to determine the different nucleotide sequence between individuals. If the polymorphism is inherited in a Mendelian fashion, then the polymorphism can be used for linkage studies and entered on the CEPH map (Hayashi, 1991; Wilton, 1993).

CEPH is the French Centre d'Etude du Polymorphisme Humain or the world database centre for mapping human polymorphisms (Dausset, et al., 1990). CEPH makes available DNA samples from a panel of reference families for the determination of genotypes for various DNA polymorphisms, which may be used for the construction of a genetic linkage map of the human genome. CEPH provides access to DNA from a common set of families for researchers investigating this area. Forty families were selected in 1984, based on large sibship size, with multigenerations to demonstrate Mendelian segregation of discovered polymorphisms. Families were chosen for their large sibship size and not for inherited diseases. The DNA of each individual was isolated from cultured lymphoblastoid cell lines (LCL). Researching collaborators investigate the genotypes with their probes, to test for segregation of these markers in the panel of families. This then contributes to the linkage analysis database and the map construction (Dausset, et al., 1990).

7.1.1 Polyacrylamide Gel Electrophoresis for Running SSCPs

Polyacrylamide gel electrophoresis is used to separate different DNA fragments. Acrylamide is an organic monomer with the following structure:



acrylamide N,N'-methylenebisacrylamide

(Sambrook, et al., 1989)

Acrylamide polymerises into long chains in the presence of free radicals, which are usually supplied by ammonium persulfate $(NH_4)_2S_2O_8$ and is stabilised by TEMED (N,N,N',N'-Tetramethylethylene diamine). Cross-linking of the chains is performed by N,N'-methylenebisacrylamide in the reaction. The porosity of the gel is determined by the length of the chains and the degree of cross-linking (Sambrook, et al., 1989).

The length of the chains is determined by the concentration of the acrylamide (between 3.5% and 20%). The greater the concentration is, the less porous and greater resolving power of the gel to separate molecules of DNA (Sambrook, et al., 1989).

The acrylamide to bisacrylamide ratio also determines the amount of cross-linking and resolving power of the gel. For example an acrylamide to bisacrylamide ratio of 29:1 is where 1 molecule of cross-linker is included for every 29 monomers of acrylamide. The less the ratio is, or the more bisacrylamide, the greater the resolving power of the gel is, as it has more cross-linking and so the gel is less porous (Sambrook, et al., 1989).

The resolving power of acrylamide gels is such that they can separate molecules of DNA that differ in length by as little as 0.2% or for example, 1 bp in 500 bp (Sambrook, et al., 1989).

7.2 MATERIALS AND METHODS

7.2.1 Amplifying Exons 1sk to 2sk in clones DNA

TPM2 primers DS 840 (exon 1sk forward) and DS 841 (exon 2sk reverse) were used on clones 2 and 4 DNA in a PCR reaction to see if the clones contained exons 1sk - 2sk of the TPM2 gene which may be used to identify a polymorphism. The reaction was set up as follows:

Reaction Mixture	1X 20 µL	7 X
Tth Buffer (5.2^{200})	5 µL	35 µL
Primers 840 + 841 (10 ng/µL of each primer)	2 µL	14 µL
Tth (5.5 u/µL)	0.1 µL	0.7 µL
H_2O	12.9 µL	90.3 µL
Total	20.0 µL	140.0 µL

Lanes 1+2	2X genomic clone 2 (10 ng/µL)	1 µL + 4 µL H_2O
Lanes 3+4	2X genomic clone 4 (15 ng/µL)	1 µL + 4 µL H_2O
Lanes 6+7	2X total human genomic DNA (10 ng/µL)	5 µL
Lane 8	1X blank (H_2O)	5 µL

The 7X reaction mixture was added with the target DNA to make a 25 µL reaction. So 20 µL of the reaction mixture was added to 1 µL of clones 2 and 4's DNA and 4 µL of H_2O. 20 µL of the reaction mixture was also added to 5 µL of the human genomic DNA and blank. Cycling and agarose electrophoresis conditions were the same as 2.2.2-4.

7.2.2 Amplifying Exon 8sk to 3' Untranslated Region of TPM2

TPM2 primers DS 870 (exon 8sk forward) and DS 867 (3'UTR reverse) were used on clones 2 and 4 DNA in a PCR reaction to see if the clones contained the 3'UTR of the TPM2 gene. Human genomic DNA was also used as a positive control. The experiment was set up as in 7.2.1, except the different primers were used.

7.2.3 TPM2 Exon 8sk - 3'UTR SSCP in 10 Unrelated Individuals

Primers DS 870 (8sk.F) and DS 867 (3'UTR.R) were used on 10 unrelated individuals to attempt to discover a polymorphism that would have an allele frequency greater than 5%. The PCR conditions used were:

For a 10 μL Reaction:-

Target DNA (50 ng)	5.0 μL	(10 ng/μL)
5X 1^{50} Buffer	2.0 μL	
(5X Tth buffer, 5 mM $MgCl_2$, 250 μM dNTP's)		
Tth DNA polymerase (5.5 units/μL)	0.1 μL	
Primer mix (870 + 867) (20 ng/μL)	1.0 μL	
(α-P^{32}) dCTP (10 μCurie/μL) (Bresatec)	0.1 μL	
H_2O	1.8 μL	
TOTAL	10.0 μL	

10X unrelated individuals DNA (10 ng/μL)	5.0 μL

PCR Cycling Conditions: 3 Step on Automatic Cycler

			time
temp	94°C	4.5 min	30 sec (denaturing)
	62°C		1 min (annealing)
	72°C		1 min (extension)
# cycles		x1	x30
	4°C		indefinitely

7.2.4 Pouring an Acrylamide Gel

1. The glass plates were thoroughly cleaned with hot water, Pyroneg detergent and a cloth.

2. The plates were rinsed by spraying 100% ethanol and they were left standing upright to dry.

3. The front plate was siliconised under a fumehood by mixing 0.5 mL of dimethyl-dichlorosilane with 5 mL of chloroform and wiping over the gel surface side of the plate with Kimwipes (lint-free tissue paper). This was so the gel would lift freely off the front plate and stay to the back plate. The back plate was lightly siliconised by wiping the moist Kimwipes over the gel surface side of the back plate. This was to ensure that the acrylamide gel poured easily and evenly between the plates.

4. The plates were assembled with the spacers and the bottom and sides of the plates were taped to prevent the gel leaking. Bulldog clamps were placed down the sides of the plates (see Figure 7.1). A plastic wedge was used to slightly separate the plates.

5. 70 mL of 12% or 6% non-denaturing acrylamide gel (39:1 acrylamide to bisacrylamide) (see Appendix 2) was de-gassed in a 100 mL beaker for 20 min, to remove the dissolved air. Otherwise fine bubbles come out of solution when the gel is poured, over the surface of the plates.

6. 100 µL of TEMED and 100 µL of 25% ammonium persulphate (APS) were added to the acrylamide and quickly mixed .

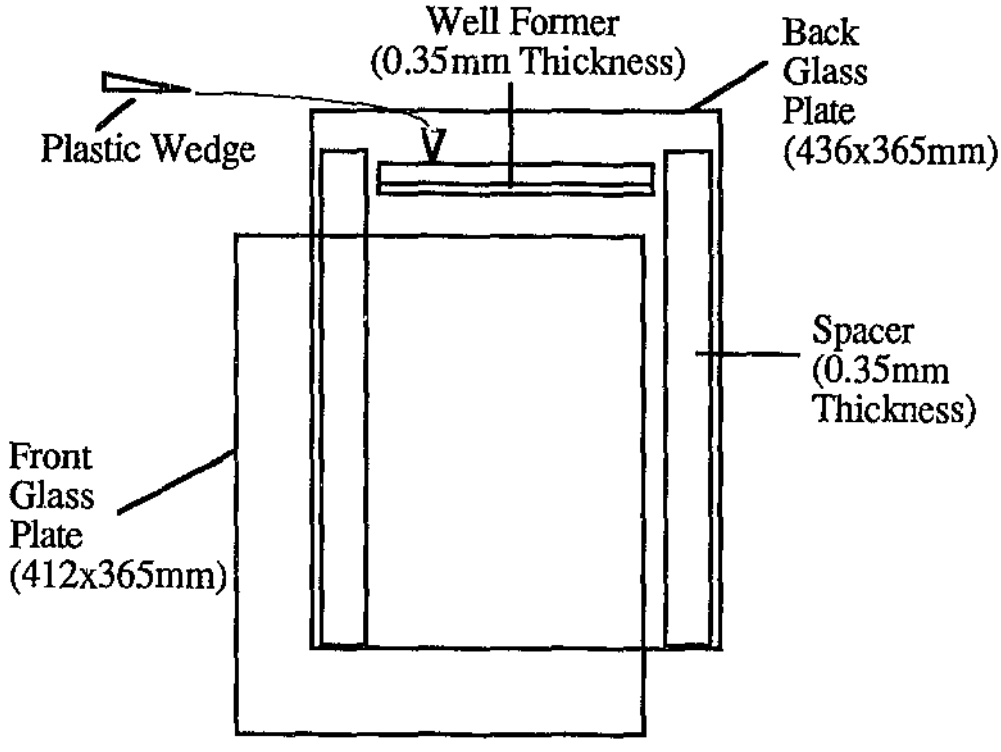
7. The plates were raised about 30° and immediately the gel mix was drawn up into a 50 mL syringe and carefully squirted between the glass plates next to the plastic wedge. If the plates were clean, the gel should move up the plates as a uniform front with no air bubbles. When the gel mix reached a few cm from the top of the plates, the plates were placed horizontal and the wedge was removed and the rest of the gap was filled by the gel.

9. Any bubbles formed were removed by a thin plastic hook (Promega).

FIGURE 7.1

Acrylamide Gel Electrophoresis

(Sambrook et al., 1989; ANRI, 1994)


Well Former
(0.35mm Thickness)
Back
Glass
Plate
(436x365mm)
Plastic Wedge
Spacer
(0.35mm
Thickness)
Front
Glass
Plate
(412x365mm)

10. A 0.35 mm thick well former was placed in the top of the plates 3/4 of the way in and clamped with bulldog clamps. Gel was poured around the well former occasionally to prevent the acrylamide from drying out and shrinking back into the plates.

11. The gel was allowed to set for at least one hour. Usually the gel polymerised within the first 5-10 min. To store the gel, damp paper towels were placed over the well former and the top of the plates was wrapped in glad wrap to prevent the gel drying out. The gel could be stored for several days at 4°C.

(ANRI, 1994; Sambrook, et al., 1989)

7.2.5 Running the Gel

1. The Hoefer Pokerface II electrophoresis equipment was used, which consisted of a lower buffer tank, an upper buffer chamber and heat distributing back plate, two plastic side clamps and electrodes.

The power pack used was the Biorad 3000Xi, which is capable of supplying constant voltage up to 1500V.

2. The clamps, paper and any glad wrap were removed.

3. The backing plate was fastened to the back glass plate with the side clamps.

4. The lower buffer chamber was filled with about 500 mL of 1X TBE, or until the buffer was a few cm above the glass plates when the gel assembly stood in the lower buffer chamber. The upper buffer chamber was filled with 1X TBE, until it was a couple of cm above the top of the front glass plate.

5. The well former was removed and a sharks tooth well comb was inserted until the teeth just entered the gel surface.

6. After the PCR reaction (7.2.3), formamide loading buffer (FLB) was added to each tube in a ratio of 1:1 (ie. 10 μL). The PCR reaction and FLB were heated to 95°C for 5 min to denature the DNA. The samples were then snap chilled on ice to form single stranded conformation fragments in the most stable tertiary form. 1 μL of each sample was loaded in each lane.

7. The electrodes were attached and the samples were run into the gel by focusing at 1250V for 1 min.

8. The samples were then run at 400V, for 16-20 hrs, at room temperature, overnight. 400V was used so the gel did not overheat and denature the single stranded conformation.

(ANRI, 1994)

7.2.6 Fixing and Drying the Gel

1. The electrodes and gel assembly were removed and the top and bottom buffer chambers were emptied down the radioactive flushing sink.

2. The gel assembly was disassembled by removing the side clamps and the backing plates.

3. The glass plates were laid down horizontally over the flushing sink and the plates separated using a metal spatula. The front glass plate was removed.

4. Strips of tissue paper were placed around the edge of the horizontal gel and fixative (10% acetic acid and 10% methanol, see Appendix 2) was poured over the entire gel. The tissue paper acted to drain away the fixative.

5. The fixative was successively poured over the gel for about 10 min so the entire gel was well covered and washed (about 500 mL in total). The tissues were removed after 10 min and the fixative was allowed to drain away. The fixative ensured the DNA would remain in the gel when vacuum blotting and stop it diffusing in the gel.

6. The gel was monitored using a geiger counter for the area of high activity and the unneeded gel (lanes not used) was cut away using a scalpel and lifted using Whatman 3MM paper.

7. A piece of Whatman 3MM paper was cut slightly larger than the remaining gel and laid over the gel, avoid trapping air bubbles. The paper was pressed down gently on the gel and lifted smoothly from one end. The gel should stick to the paper and come away flat.

8. The gel was covered with glad wrap and cut to the size of the paper. One corner was cut to orientate the gel.

9. The gel lift was laid on a piece of 3MM in the gel drier and covered with the silicon rubber seal. The vacuum was turned to full and the gel dried for one hour at 80°C.

10. Autoradiography was performed as usual with Dupont Cronex 4 X-ray film with Quanta III autoradiography intensifying screens at -80°C, for an empirically determined time.

(ANRI, 1994)

7.2.7 TPM2 8sk - 3'UTR SSCP on DNA from 97 Unrelated Individuals

Primers DS 870 and 867 were used on 97 unrelated individuals to determine the allele frequency of the polymorphism, using the methods described in 7.2.2-6. The samples were run on a 6% non-denaturing acrylamide gel.

7.2.8 TPM2 8sk - 3'UTR SSCP on DNA from 9 Families to Determine if Polymorphism Segregates in a Mendelian Fashion

For those of the 97 individuals that had the rare allele, families were selected, based on multigenerations to see if the polymorphism segregated in a Mendelian fashion. Nine families were selected and primers DS 870 and 867 were then used on these families as in 7.2.2-6.

7.2.9 Sequencing of the TPM2 Polymorphism from Exon 8sk.F-3'UTR.R from Band Stabbing an Acrylamide Gel.

The ~250 bp TPM2 STS (8sk-3'UTR) samples that were homozygous for the rare allele were run together side by side on a 6% non-denaturing acrylamide gel, and the samples for the common allele were run side by side, to compare all the alleles to see if there were more than two alleles. The gel was exposed wet, so it wasn't vacuum dried. The lifted gel was labelled with fluorescent ink and the autorad was then matched back with the gel. A flame heated iron needle was then pushed through the autorad and into the gel to mark where rare and common alleles for several individual samples were. For each individual sample, the four corners of the lane where the allele was present was stabbed. The bands of 7 heterozygous individuals for the rare allele were cut out with a scalpel and placed into separate 1.5 mL eppendorf tubes with 0.5 mL of TE buffer. The gel bands for four individuals, that were homozygous for the common allele, were cut out.

The gel with the cut out bands was then re-exposed to confirm that the bands were cut out exactly where the rare and common alleles were.

Using the DNA eluted from the bands as template, a 50 µL PCR was set up using primers DS 870 (exon 8sk.F) and DS 867 (exon 3'UTR.R) as in 2.2.6.1, except 5X 2^{100} (10 mM $MgCl_2$, 500 µM dNTP's) buffer was used. Three individuals for the rare allele and one individual for the common allele were amplified. 5 µL of the eluted

template was used with 20 µL of the reaction mixture. The cycling conditions were: 94°C for 5 min, 30 cycles of 94°C for 30 sec and 60°C for 6 min. 3 µL of each product was run on a 2% agarose gel, at 100V, for 1 hr, with 3 µL of λ*Pst* I marker. The DNA from each sample was then run on a low melting point agarose gel and purified using Wizard™ PCR Preps Purification System, as in 2.2.6.1-2. The DNA concentration was then measured on the spectrophotometer.

50 ng each of the four samples were then used in the sequencing reaction as in 2.2.8. Sequencing was from primer DS 870 (exon 8sk-forward) and from primer DS 867 (3'UTR-reverse) for each sample.

7.2.10 Sequencing Homozygous Individuals for the TPM2 (8sk.F-3'UTR.R) common and rare allele

After screening the 97 individuals and 9 families, two individuals were discovered to be homozygous for the rare allele. The DNA from these two individuals were amplified using primers DS 870 and 867 and the polymorphism sequenced as in 7.2.8. The sequence was compared by sequencing two homozygous individuals for the common allele.

7.2.11 Restriction Digest of ~250 bp TPM2 STS (8sk-3'UTR)

After finding a restriction site for the suspected polymorphism base change, a restriction digest was set up using *Bgl* I. A PCR was performed using primers DS 870.F (exon 8sk) and DS 867.R (3'UTR) on total human genomic DNA from a heterozygous allelic individual, a homozygous common allele individual and a homozygous rare allele individual. The subjects were chosen by looking at the SSCP gels. The conditions of the PCR were:

For a 25 µL Reaction:-

Reaction Mixture		**Final Conc.**
Target Genomic DNA (50 ng)	5.0 µL	2 ng/µL
5X 1^{100} Buffer (buffer, nucleotides and $MgCl_2$)	5.0 µL	1 mM, 100 µM
(5X Tth buffer, 5 mM $MgCl_2$, 500 µM dNTP's)		
Tth DNA polymerase (Biotech) (5.5 units/mL)	0.1 µL	0.22 u/µL
Primer mix (10 ng/µL of each primer)	2.0 µL	0.8 ng/µL
H_2O to 25 µL	12.9 µL	
TOTAL	25.0 µL	

Target DNA		
1X heterozygous allelic individual	(10 ng/µL)	5 µL
1X homozygous common allelic individual	"	"
1X homozygous rare allelic individual	"	"

PCR Cycling Conditions: 2 Step on Automatic Thermal Cycler

			time
temp	94°C	5 min	30 sec (denaturing)
	60°C		6 min (annealing and extension)
# cycles		x1	x30
	4°C		indefinitely

The STS product from the PCR was run on a 2% agarose gel at 100V, for 1 hour as in 2.2.3-4.

The STS products from the three different individuals were digested using the restriction enzyme *Bgl* I, as in 5.2.9, and run on a 3% agarose gel at 100V for 1 hr and 20 min.

The gel was stained with ethidium bromide (0.5 μg/mL) for 10 min and observed on the UV transilluminator as in 2.2.4. pGem marker was used to determine fragment sizes.

7.3 RESULTS AND DISCUSSION

7.3.1 Amplifying Exons 1sk to 2sk in clonal DNA

TPM2 primers DS 840 (exon 1sk forward) and DS 841 (exon 2sk reverse) were used on clones 2 and 4 DNA in a PCR reaction to see if the clones contained exons 1sk - 2sk of the TPM2 gene, which may be used for an SSCP. Clones 2 and 4, had DNA that was specific to TPM2 and so was used as target DNA. The clones were used, as the primers did not amplify a product when total human genomic DNA was used and by having more specific template would increase the probability of the PCR working. There are about 200 bp between the primers using Clayton et al.'s (1988) structure of TPM3, in Figure 1.3, 100 bp in the muscle specific exons and 100 bp in the intron. An approximate 200 bp fragment was expected from TPM2, but there were no discernible bands from the amplification when run on a 2% agarose gel (no data shown). This meant that clones 2 and 4 most likely did not contain exons 1sk and 2sk.

The average insert of the TPM2 clones was 15-18 kb, which were already shown to contain exons 6 - 8sk, in Figure 5.9. Since the TPM3 gene is approximately 42 kb long, or 28 kb from exon 1sk-8sk and assuming it has a similar structure to TPM2, then it was expected that these clones would most likely not contain these exons (1sk - 2sk) because of the limitations on the amount of DNA that can be cloned in the EMBL3 SP6/T7 vector (Sambrook, et al., 1989). But it was still worth attempting to confirm this hypothesis.

7.3.2 Amplifying Exon 8sk to 3'UTR of TPM2

TPM2 primers DS 870 (exon 8sk forward) and DS 867 (3'UTR reverse) were used on clones 2 and 4 DNA in a PCR reaction to see if the clones contained the 3'UTR of the

TPM2 gene. Human genomic DNA was also used as a positive control. In Figure 7.2, using the λPst I marker, it can be seen an approximate 250 bp product was amplified in clone 2 (lanes 1&2), clone 4 (lanes 3&4) and in human genomic DNA (lanes 5&6). There are 259 bases from and including primer 870 (in exon 8sk) to primer 867 (in 3'UTR) using the published sequence of (Widada, et al., 1988). Therefore, there is no 4 kb intron VIII, before exon IXsk, which is in the 3'UTR as Clayton et al., (1988) suggests in TPM3, for the skeletal muscle isoform (see Figure 1.3). This would make the TPM2 gene at least 4 kb less than the 42 kb TPM3, so the TPM2 gene would be approximately 38 kb.

One can be confident that the amplified ~250 bp TPM2 STS (8sk-3'UTR) was not a pseudogene, because it was amplified from the two clones and because primer DS 870 was in a muscle specific exon (2.1.2.2). These clones contained a fragment of the TPM2 gene in the insert, as had already been shown by sequencing and both were localised and the primers could only anneal to the target DNA present. The STS was repeated from the human genomic DNA, as a positive control, so it can be confident the STS was unique to TPM2.

7.3.3 TPM2 Exon 8sk - 3'UTR SSCP in 10 Unrelated Individuals

Primers DS 870 (8sk.F) and DS 867 (3'UTR.R) were used on 10 unrelated individuals to attempt to discover a polymorphism that would have an allele frequency greater than 5%. In Figure 7.3, it can be seen in lanes 1 and 3, there was a SSCP. These individuals were heterozygous for that allele. The other individuals were all homozygous. The products were run on a 12% non-denaturing acrylamide gel at 400V for 24 hours. The gel was exposed for 8 hrs at -80°C with an intensifying screen. Lanes 11 and 12 were a positive control. The image is not clear, but the PCR and electrophoresis conditions were optimised later.

FIGURE 7.2

~250 bp TPM2 STS (Exons 8sk - 3'UTR) from Clones 2, 4 & Human Genomic DNA

Clone 2
Clone 4
Genomic DNA
Another Experiment
λ Pst I
~500bp TPM2 STS
(exons 8sk-3'UTR)

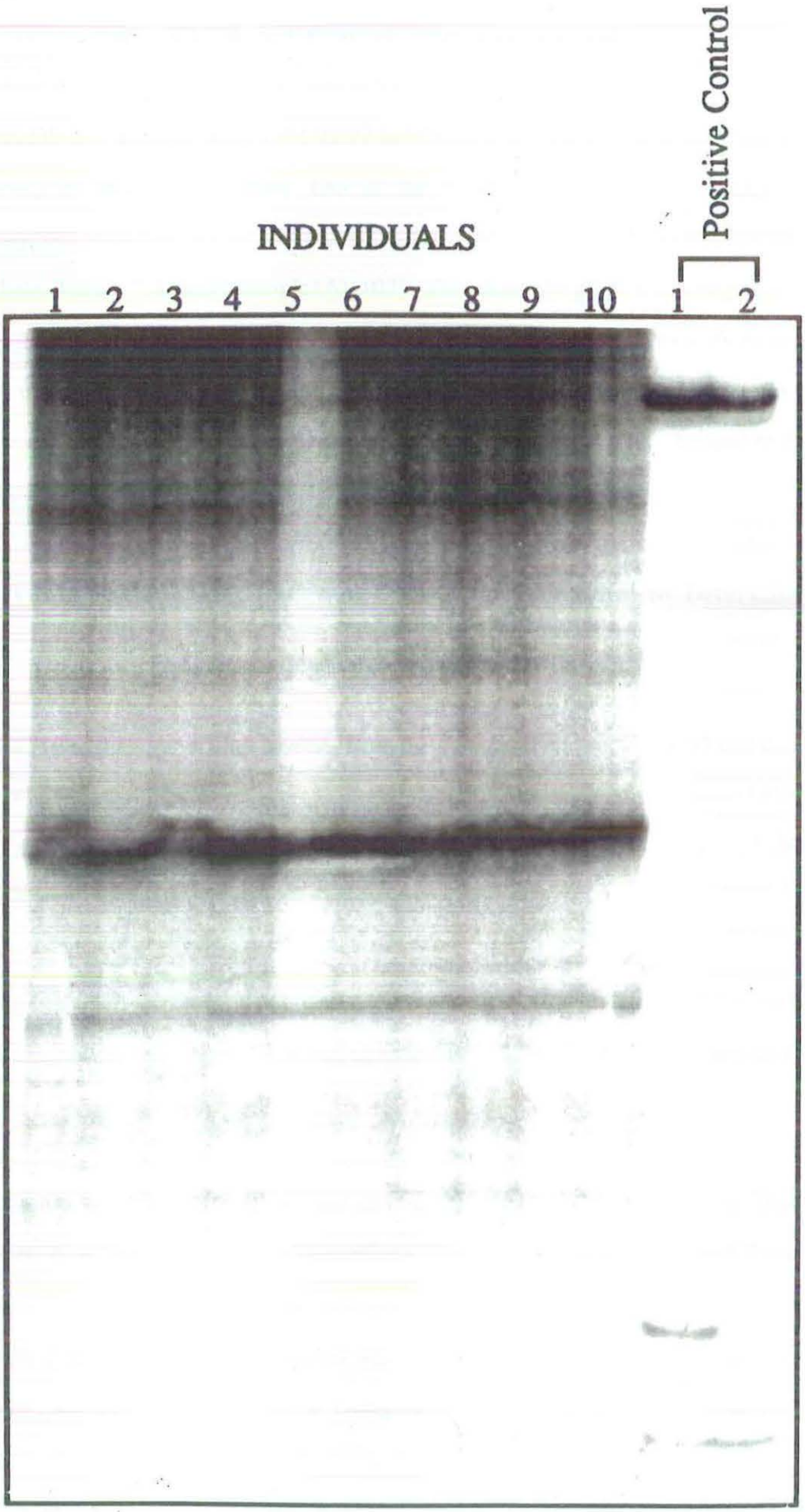
FIGURE 7.3

SSCP of DNA from 10 Unrelated Individuals from the ~250 bp TPM2 STS (exon 8sk - 3'UTR)

12% SSCP Gel, Run at 400V for 24 hrs at Room Temperature.

Developed After 8 hrs, at -80°C, 1 Quanta III Intensifying Screen.

Annealing Temperature 55°C


INDIVIDUALS
1 2 3 4 5 6 7 8 9 10
Positive Control
1 2

7.3.4 TPM2 8sk - 3'UTR SSCP in 97 Unrelated Individuals

Primers DS 870 and 867 were used on 97 unrelated individuals to determine the allele frequency of the polymorphism. Out of the 97 individuals, 16 individuals were heterozygous with both alleles and one individual (sample 85) was homozygous for the rare allele (Figure 7.4 shows samples 61-103). Therefore, the allele frequency was 0.09 for the rare allele, 0.91 for the common allele and the heterozygosity was 0.16. Note, the SSCP running conditions were improved as a 6% non-denaturing acrylamide gel was used to separate the alleles more and an annealing temperature of 62°C, instead of 55°C was used, which increased the annealing specificity of the primers.

7.3.5 TPM2 8sk - 3'UTR SSCP on DNA from 9 Families to Determine if Polymorphism Segregates in a Mendelian Fashion

The SSCP was then run in nine families from the 17 individuals out of the 97 that had the allele. The nine families were selected predominantly to demonstrate the segregation of alleles in a Mendelian fashion, so as many generations as could possibly be included in the family was the main criterion, as well as large sibship size.

The segregation of the rare and common alleles was demonstrated in a Mendelian fashion in all nine families except one. The segregation data will be shown for just two of these families.

From Figure 7.5 showing the SSCP gel and family 1's pedigree, it can be seen that the rare allele does not segregate in a Mendelian fashion. The granddaughter and grandson (samples 5 & 6 respectively) are heterozygous for the alleles yet neither of their parents (samples 3 & 4) are heterozygous, but are both homozygous for the common allele. Likewise, the mothers grandparents (samples 1 & 2) are both homozygous for the common allele. It was later demonstrated by microsatellite linkage analysis at Royal Perth

FIGURE 7.4

SSCP Frequency - Unrelated Individuals 61 - 103.

6% SSCP Gel Run at 400V, for 16.5 hrs at Room Temperature.

Developed after 4 hrs, at -80°C with 1 Quanta III Intensifying Screen.

Annealing Temperature 62°C.

INDIVIDUALS

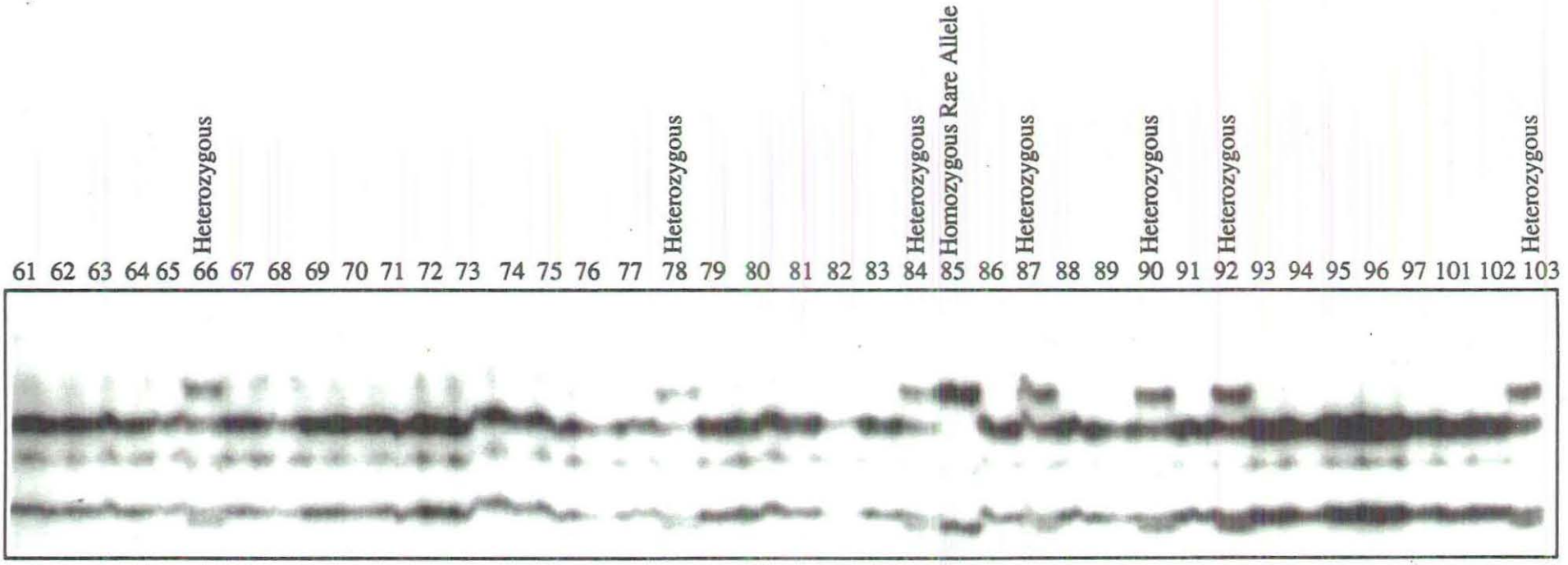
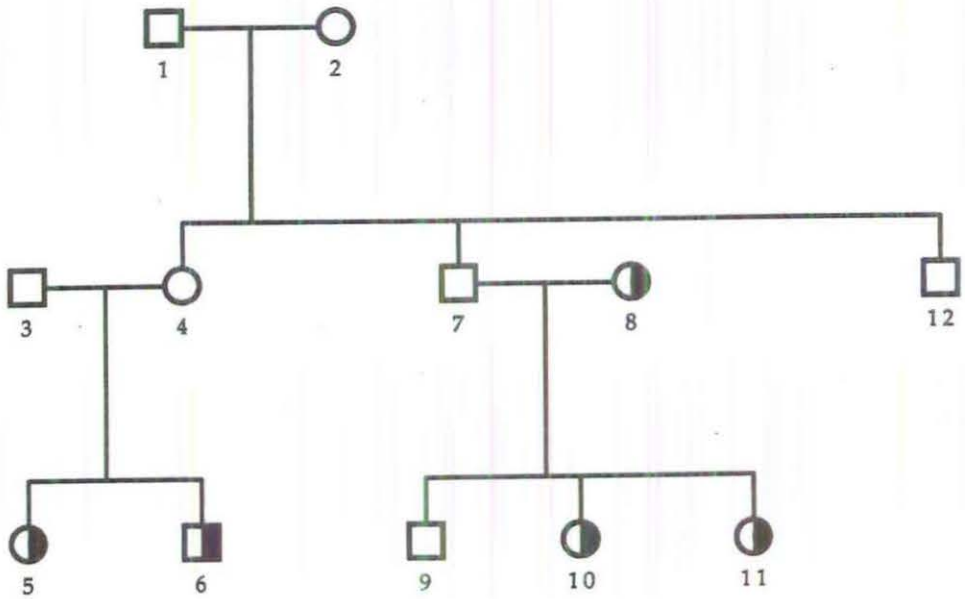


FIGURE 7.5

Segregation of Polymorphic Alleles in Family 1.

6% SSCP Gel Run at 400V for 18.5 hrs at Room Temperature.

Developed after 3 hrs, at -80°C with one Quanta III Intensifying Screen.


1
2
3
4
5
6
7
8
9
10
11
12

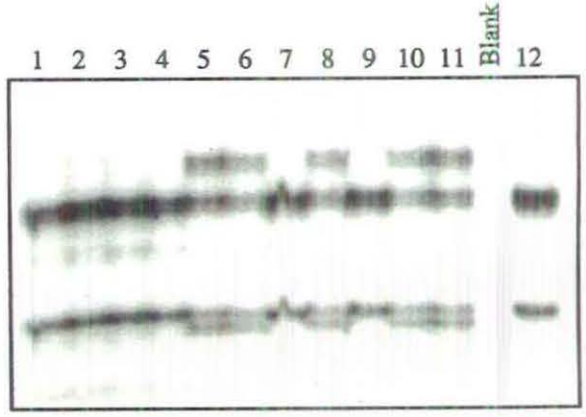
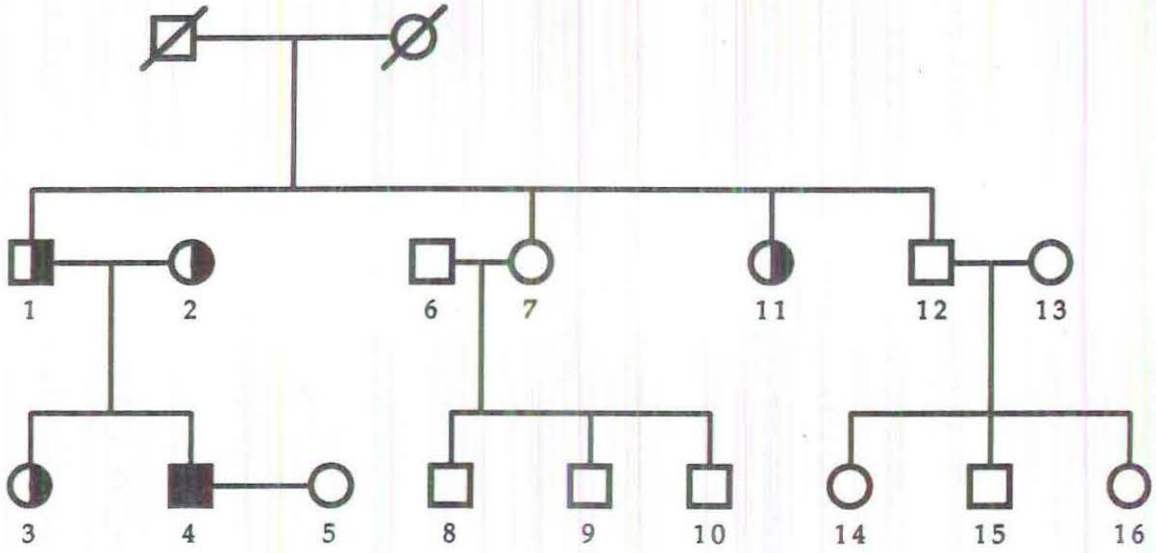

1 2 3 4 5 6 7 8 9 10 11 Blank 12

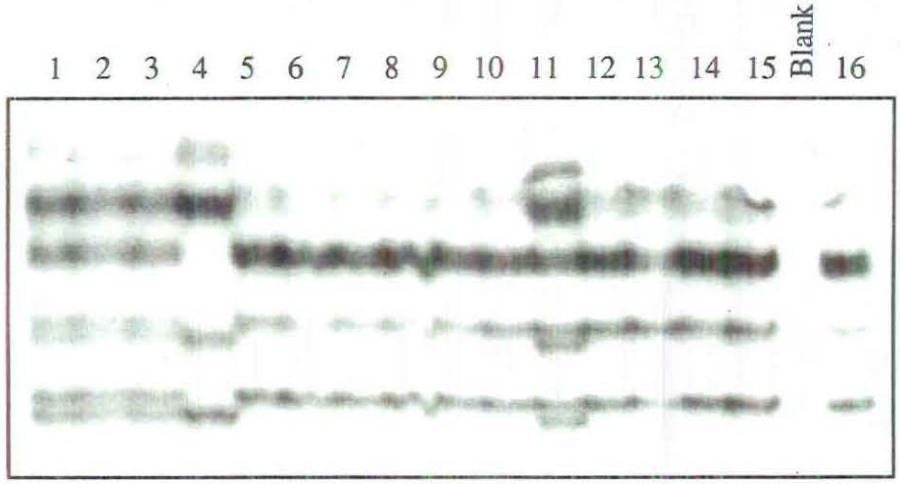
FIGURE 7.6

Segregation of Polymorphic Alleles in Family 2

6% SSCP Gel Run at 400V for 17.5 hrs at Room Temperature.

Developed after 1.5 hrs, at -80°C, with one Quanta III Intensifying Screen.


1
2
6
7
11
12
13
3
4
5
8
9
10
14
15
16


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Blank 16

Hospital, that there was a sample mix-up in the supplied DNA and that the father (sample 3) was really the son (sample 6), which then shows the rare allele segregating in the family. This was quite a useful error to discover, as any other research trying to demonstrate Mendelian segregation in this family, would also not have been able to do so. It also demonstrates the polymorphism is real and segregates in families as it was able to detect a sample mix-up, when blood samples were taken. Therefore, Family 1 was very useful for demonstrating the segregation of the TPM2 polymorphic alleles.

Family 2 (Figure 7.6) was interesting, because two heterozygous parents for the TPM2 8sk - 3'UTR allele (samples 1 & 2) produced a daughter that was heterozygous (sample 3) and a son who was homozygous for the rare allele (sample 4). The homozygous allele can be seen quite clearly in Figure 7.6, sample 4 for Family 2.

It can therefore, be stated confidently that the TPM2 8sk-3'UTR allelic polymorphism segregates in a Mendelian fashion.

The PCR and electrophoresis conditions were optimised for the SSCP and the conditions listed in 7.2.3 were discovered to be optimal, when run on a 6% non-denaturing acrylamide gel, at 400V, for 20 hours. Note, the annealing temperature 62°C was quite high compared to normal PCR annealing temperatures.

7.3.6 Sequencing of the TPM2 Polymorphism from Exon 8sk -3'UTR from Band Stabbing an Acrylamide Gel.

An SSCP gel of the first 55 unrelated individuals was run on a 6% non-denaturing acrylamide gel at 300V for 19 hours (see Figure 7.7). The PCR conditions were the same as 7.2.3. Samples 1, 3, 22, 26, 33, 41, 45, and 51 were heterozygous for the allele and were run together, to see clearly if there were only two different alleles. There were only two alleles present and the rare alleles for these heterozygous individuals were cut out using the band stabbing technique described in 7.2.9. The sample eluates were PCR

FIGURE 7.7

Band Stab Gel of Rare Allele from Heterozygous Individuals and Common Allele from Homozygous Individuals.

6% SSCP Gel run at 300V, for 19 hrs at Room Temperature.

Developed after 29 hrs, at Room Temperature with No Intensifying Screens.

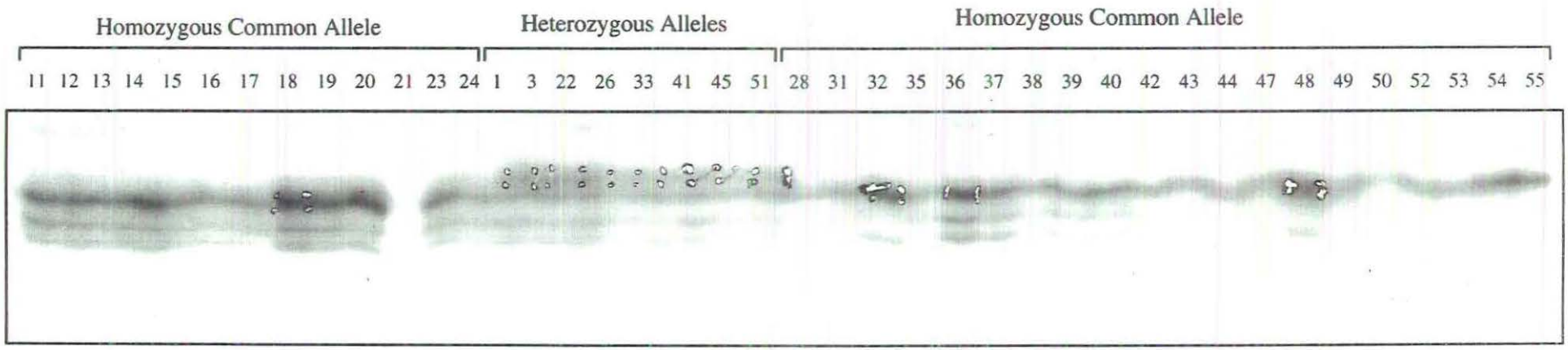

Homozygous Common Allele
Heterozygous Alleles
Homozygous Common Allele
11 12 13 14 15 16 17 18 19 20 21 23 24 1 3 22 26 33 41 45 51 28 31 32 35 36 37 38 39 40 42 43 44 47 48 49 50 52 53 54 55

FIGURE 7.8

Band Stab Gel Re-exposed to Confirm Exact Alleles were Stabbed.

Developed after 2 days at Room Temperature with No Intensifying Screens.

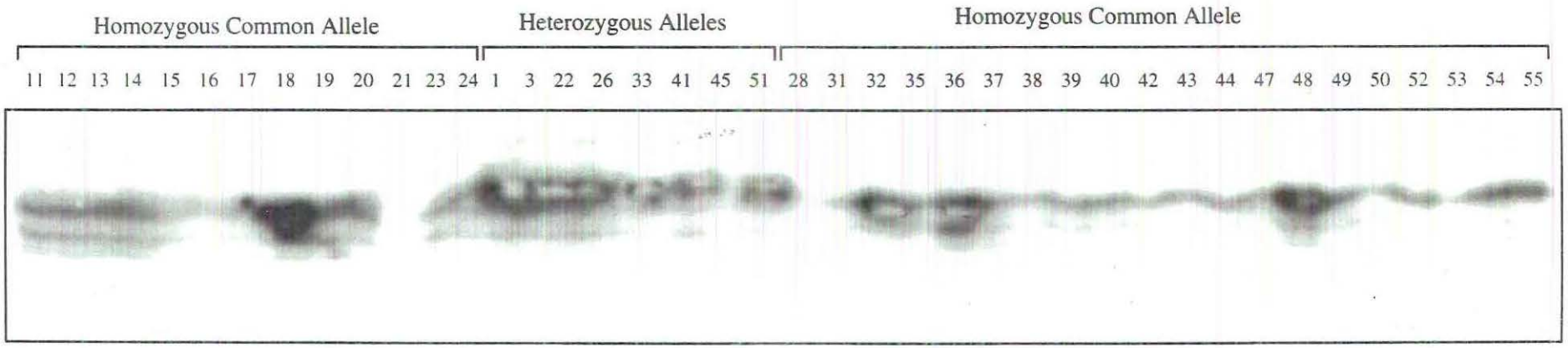

Homozygous Common Allele
Heterozygous Alleles
Homozygous Common Allele
11 12 13 14 15 16 17 18 19 20 21 23 24 1 3 22 26 33 41 45 51 28 31 32 35 36 37 38 39 40 42 43 44 47 48 49 50 52 53 54 55

TABLE 7.1

Sequence of TPM2 SSCP from Gel Band Stabs

TABLE 7.1

SEQUENCE OF TPM2 SSCP BAND STABS

clive2 22/12/94

	10	20	30	40	50
1. Band Stab Ind3 870F)R			NNNNCTC	ATTNCGAGNC	ATGGCCATTA GCGAGGAACT
2. Band Stab 41 870F)A A			NNTN	CNTNCTAGTN	ATGGCCATTA GCGAGGANCT
3. Band Stab 51 870F)R L			NCCCGC	ACCNCNGNGA	ANAAATNAGA GNAGAAATGG
4. Family 2(4) 870F)E L				TANNGNATG	TCTTTNGANA CGNCCTGGCA
5. Family 2(4) 867R) E	TTAAAATCTA	TGCCCAGTAG	ATGAAGTACA	GAGGCCANTA	GCGANGGNCT
6. Band Stab 48 870F)C L				ANGN	ACGGNCATTA GCGAGGAACT
7. Family 1(3) 870F)O E	Bad Sequen	ce		NTTAANNN	ATNCTATANA CAAGGTACNN
8. Family 1(3) 867R)M	TTAAAATCTA	TTCCCAGTAG	NTGAAGTNCA	AGGCCATTAA	CGAGGANTCT
9. TPM2(Widada et al.))M	AATCT	ATGCCCAGAA	GATGAAGTAC	AAGGCCATTA	GCGAGGAACT
O	^G-	Addition			
N					

	60	70	80	90	100
1. Band Stab Ind3 870F)R	GGACAACGCA	CTCAATGACA	TCACCTCCCT	CTGAGCCCCA	CACCAGNGTG
2. Band Stab 41 870F)A A	GGACAANGCA	CTCAATGAGA	TCACCTNCCT	CTGGGTCCCA	CACCAGNGTG
3. Band Stab 51 870F)R L	ACAGNGCATT	CAACTGCANA	TCACNTCCCT	CTGAGTCCCA	CACCAGCGAG
4. Family 2(4) 870F)E L	NTAGCACTCA	TCNGACATCA	CCTGCCACTC	TGNNACCCCA	CACCAGNGTN
5. Family 2(4) 867R) E	GGACAACACT	NTCNATGACA	TCACCTCCCT	CTGATCCCNA	CACCAGCGTG
6. Band Stab 48 870F)C L	GGACAACGCA	CTCAATGACA	TCACCTCCCT	CTGAGCCCCA	CGCCAGCGTG
7. Family 1(3) 870F)O E	NCTATAGGAN	NCANTGACAT	CACCTCNACT	CTGAANCNCA	CGAGCAGGGA
8. Family 1(3) 867R)M	GGACNACNCN	CTCAATGACA	TCACCTCCCT	CTGANCCCCA	CGCCAGCGTG
9. TPM2(Widada et al.))M	GGACAACGCA	CTCAATGACA	TCACCTCCCT	CTGAGCCCCA	CGCCCAGCGT
O				P olymorphis	m^ ^Add-C
N					

	110	120	130	140	150
1. Band Stab Ind3 870F)R	GGCANCTCAG	CTCTCTTCTC	TCCTCTCCTT	TCCATTCTCT	CTATGGGGAG
2. Band Stab 41 870F)A A	GGCAACTCAG	AGTCTCTNCT	CTCNTCTCCT	TCCCATTCTC	TCTNTGGGGA
3. Band Stab 51 870F)R L	GGCAGCTNAG	NNATCTCCTN	TCTTNTCCTT	CCCATNCTCT	CTATGGAGAG
4. Family 2(4) 870F)E L	GGCAGCNCAG	GTCTCTTCTC	TCCTCTCCNN	GCNANNCTCT	CTATGGGGAG
5. Family 2(4) 867R) E	GCCACCNCAT	CTCTCTTCTC	TCCTCNCCTT	TCCATNCTCN	CTATGGGGAG
6. Band Stab 48 870F)C L	GGCACCTCAG	CTCTCTTCTC	TCCTCTCCTT	TCCATTCTCT	CTATGGGGAG
7. Family 1(3) 870F)O E	ATGACACCNC	AGATCTCTGT	CTCTCCTCTC	CTTTCCATCC	TCTCTATGGG
8. Family 1(3) 867R)M	NCCACCTCAG	CNCTCTTCTC	TCCTCTCCTT	TCCATTCTCT	CTATGGGGAG
9. TPM2(Widada et al.))M	GCCACCTCAG	CTCTCTTCTC	TCCTCTCCTT	TCCATTCTCT	CTATGGGGAG
O	^^Del-G &	Sub-G->C			
N					

	160	170	180	190	200
1. Band Stab Ind3 870F)R	GGGAGCAGNN	AGGAGGAGCA	GAAATTGNCA	ACATTGNACA	GNCAGGCTGG
2. Band Stab 41 870F)A A	GGGGAGGAGA	GAGGAGGAGT	AGAAATNGCC	AATATTGNAN	AGNCANTGTN
3. Band Stab 51 870F)R L	GGGAGCGAGG	NAGGAGGAGC	AGAAGTTGTA	GCCATNGANN	GAGACAGGCA
4. Family 2(4) 870F)E L	GGGAGCAGGN	AGGAGGAGCA	GAANTTGGCA	GCATTGAACA	GTCAGGTTGG
5. Family 2(4) 867R) E	GGGANNAGGC	AGGAGGANCA	GAAGTTNCCT	ANNATTCACA	ACCNGGCANN
6. Band Stab 48 870F)C L	GGGAGCAGGC	AGGAGGAGCA	GAAATTGCCA	ACATTGCACA	GCCAGGCTGG
7. Family 1(3) 870F)O E	GAGGGGAGCA	GGCAGGAGGA	GNAGAAATTG	ACANCATTGA	ACAGTCAGGT
8. Family 1(3) 867R)M	GGGANNAGGC	NGGAGGAGCA	GAAATTGCCT	NCNATNCACA	TCCNGGCTAG
9. TPM2(Widada et al.))M	GGGAGCAGGC	AGGAGGAGCA	GAAATTGCCA	ACATTGCACA	GCCAGGCTGG
O	^AG-D	eletion			
N					

	210	220	230	240	250
1. Band Stab Ind3 870F)R	GAGGAGNCTA	GGGAGAGCCC	CCATCATGAC	CACCACCCAC	TCTGGNACTG
2. Band Stab 41 870F)A A	NGNAGGAGCG	GGGGAGAGGC	ACACATCATN	ACCACCATCA	NCTCCTGNAC
3. Band Stab 51 870F)R L	NGGGNGCAGT	AGAGTGAGNG	AGAGCCNTCN	ATGATNACCC	ACCNACCATT
4. Family 2(4) 870F)E L	GGGTAGCCTA	GGGAGAGCCC	CCATCATGNC	CACCACCCAC	TCTTANACTG
5. Family 2(4) 867R) E	GANCAGCNTA	AGAGAGANCC	CCTNTNATAC	NTTATCCACA	CTANNGGNGN
6. Band Stab 48 870F)C L	GAGCAGNCTA	GGGAGAGCCC	CCATCATGCC	CACCACCCAC	TCTGGCACTG
7. Family 1(3) 870F)O E	TGGGGGTAGT	CTAGGGAGAG	NCCCCNTCAT	GNCCACNTCC	CACTCTGGGA
8. Family 1(3) 867R)M	GATCATCCAA	GAGAGAACCC	CTAGAATNTC	CNCTATCCAC	TCTNNNNNGC
9. TPM2(Widada et al.))M	GAGCAGCCTA	GGGAGAGCCC	CCATCATGCC	CACCACCCAC	TCTGGCACTG
O					
N					

TABLE 7.2

Sequence of TPM2 SSCP Homozygous Alleles

TABLE 7.2
SEQUENCE OF TPM2 SSCP HOMOZYGOUS ALLELES

Clive SSCP2 22/12/9

1. Family 2(4)	870F	)R	
2. Family 2(4)	867R	)A	A
3. Individual 85	870F	)R	L
4. Individual 85	867R	)E	L
5. Family 2(12)	870F	)C	E
6. Family 2(12)	867R	)O	L
7. Band Stab In48	867R	)M	E
8. TPM2(Widada et al.)		)M	
		M	
		O	
		N	

```
                    10        20        30        40        50
                     |         |         |         |         |
                                                AGTCANGG CCATTAGCGA GGAACTGGAC
GAGTCTATGC CCAGAAGATG AAGTGCAAGG CCATTAGCGA GGAACTGGAC
                                                AGNACAAGG CCATTAGCGA GGAACTGGAC
AAGTCTATNC CCAGGAGATG AAGNACGAGN CCATTAGCGA GGAGCTGGGC
                                                AGCCAAGGC CATTAGCGAG GNAACTGGAC
Bad Sequen ce     ATGN AGANACANGG CGGTTAGNGA TGANCANGAC
NAATCTATTC CCAGNAGATG AAGTACAAGN CCATTAGCGA GGNACTGGAC
AAGTCTATGC CCAGAAGATG AAGTACAAGG CCATTAGCGA GGAACTGGAC

                    60        70        80        90       100
                     |         |         |         |         |
AACGCACTCA ATGACATCAC CTCCCTCTGA GCCCCACACC AGCGTGGCCA
AACGCACTCA ATGANATNAC CTCCCTCTGA GCCCCACACC AGCGTGNCCA
AACGCACTCA ATGACATCAC CTCCCTCTGA GCCCCACACC AGTGTGGCCA
ANCGCACTCA ATGACATCAC CTCCCTCTGA GCCCCACACC AGCNTGGCCA
AACGCACTCA ATGACATCAC CTCCCTCTGA GCCCCACGCC AGCGTGGCCA
GACTNANACA ATAANAACAC CTCCCTCTNA ACCCAACTCC ATCNTGTCCA
AACGCACTCA ATGACATCAC CTCCCTCTGA GCCCCACACC AGCGTGGCNA
AACGCACTCA ATGACATCAC CTCCCTCTGA GCCCCACGCC CAGCGTGCCA
                                Polym orphism^   ^AddC ^Del

                   110       120       130       140       150
                     |         |         |         |         |
CCTCAGCTCT CTTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
CCTCAGCTCT CTTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
CCTCAGCTCT CTTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
CCTCAGCTCT CTTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
CCTCAGCTCT CTTCTCTCCT CTCCTTNCAA TTCTCTCTAT GGGGAGGGGA
CCTCAACTCT CGTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
CCTCAGCTCT CTTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
CCTCAGCTCT CTTCTCTCCT CTCCTTTCCA TTCTCTCTAT GGGGAGGGGA
-G

                   160       170       180       190       200
                     |         |         |         |         |
GCAGGCAGGA GGAGCAGAAA TTGCCAACAT NGCACAGNCA GGCTGGGAGC
GCAGGCAGGA GGAGCAGAAA TTGCCAACAT TGCACAGCCA GGCTGGGAGC
GNAGGTAGGA GGAGCAGAAN TTGTCAACAT NGCACAGCCA GGTTGGGAGN
GCAGGCAGGA GGAGCAGAAA TTGCCAACAT TTCACAGCCA GGCTGGGAGC
GCAGGNAGGA GGAGNAGANN TTGNCACCAT CGNACAGCCA GGTTGGGAGC
GCAGNCAGGA GGAGCAGAAA TTNCCAACAT TTCACANCCA GACTAAGAAC
GCAGGCAGGA GGAGCAGAAA TTGCCAACAT TGCACAGCCA GNCTGGGAGC
GCAGGCAGGA GGAGCAGAAA TTGCCAACAT TGCACAGCCA GGCTGGGAGC
 ^AG-Delet ion

                   210       220       230       240       250
                     |         |         |         |         |
AGCCTAGGGA GAGNCCCCAT CATGCCCACC ACCCANTCTG GTACTGGCTT
AGCCTAGGGA GAGCCCCCAT CATGCCCACC ACCCACTCGT
AGCCTAGGGA GAGCCCCCAT CATGCCCACC ACCNACTCTG GNACTGGNTT
AGCCTAGGGA GAGCCCCCAT CATGCCCACC ACCCACTCGT
AGCCTAGGGA GAGCCCCCAT TATGCCCACC ANCCACTCTG GCACTGGCTN
ANCCTAGGGG GGNNCCCCNA AAGATNCCCA CCACCCACTC NTANGTNANG
AGCCTAGGGA GAGCNCCCAT CATGCCCACC ACCCACTCGT
AGCCTAGGGA GAGCCCCCAT CATGCCCACC ACCCACTCTG GCACTGGCTT
```

amplified and sequenced. Samples 18, 32, 36, 48 that were homozygous for the common allele were also cut out and amplified to compare the sequence between the two alleles. The gel was then re-exposed to show if the exact alleles had been cut out. Figure 7.8, shows that the exact alleles were cut out.

Table 7.1 shows the sequence from the band stab amplifications. The samples were sequenced from primer DS 870 (8sk.forward) and also from primer DS 867 (3'UTR.reverse):

Lines

1-3. Heterozygous Individuals 3, 41, 51 } Band stabs of rare allele

4-5. Family No. 2 (sample No.4) } Homozygous individual for the rare allele

6. Homozygous Individual 48 } Band stab of homozygous common allele

7-8 Family No. 1 (sample No. 3) } Homozygous individual for common allele

9. TPM2 sequence (Widada, et al., 1988) } Published sequence

The band stabs were only sequenced from primer DS 870, exon 8sk forward. Any samples that were sequenced in the reverse direction (primer DS 867.R) were rotated and the complementary sequence was given in Table 7.1, using SeqEd^{TM} 675 DNA Sequencer Editor (ABI). This was to compare the sequencing data from the reverse primer, with the forward primer.

There was a sequence difference between the rare and common alleles at base 92 where all the rare alleles had an adenine base and Widada et al. and the common alleles had a guanine base. The sequencing for individual 473 (7) DS 870.8skF was quite bad though. The sequencing was not ideal, as there were a lot of base changes between the samples.

It was noticed however, that the sequence from the samples in some places consistently differed to (Widada, et al., 1988) published sequence in the 3'UTR. At base 93-96

Widada's sequence had a CCCA, while the samples mostly just had a CCA, so Widada's sequence had an extra C or insertion, which misaligned the bases until Widada has a G deletion at 101-102 and a C substituted for a G, so the sequence is realigned after base 103 (see Table 7.1).

```
                    3'UTR
                91          101
Widada et al    CGCCCAGCGT GCCACCT
Sequencing      CACCAGCGTG GGCACCT
                     *       **
```

It was also noticed that Widada had an extra AG at bases 156-157, which were not present in 6 out of the 8 samples. The other samples (Family 2 (4)-867.R and Family 1 (3)-867.R) had bad sequencing at this point. These two bases were not included in the TPM2 sequence of Widada et al. in Table 7.1, as it would have meant the comparison and alignment of TPM2's sequence with the other samples would be difficult to follow, but it is shown below:

```
                    3'UTR
                151         161
Widada et al    GGGAGAGCAG GCAGGAG (AG inserted)
Samples         GGGAGCAGGC AGGAGGA
                     **
```

So there were five consistent changes in the sequence data from that of Widada et al.'s published sequence, all in the 3'UTR, and a highly probable cause for the TPM2 SSCP polymorphism.

7.3.7 Sequencing Homozygous Individuals for the TPM2 (8sk - 3'UTR) common and rare allele

Another homozygous individual for the rare allele was discovered when screening the 97 individuals. The most direct method for sequencing the polymorphism was now therefore to amplify the DNA of the two individuals that were homozygous for the rare allele and sequence the fragment. The sequence could then be compared to amplified DNA from individuals that were homozygous for the common allele.

The samples were sequenced using primers DS 870 (8sk.F) and 867 (3'UTR) and the results are shown in Table 7.2:

Lines 1-2 Family No. 2 (sample No.4)	} Homozygous rare allele TPM2 exon 8sk-3'UTR
Lines 3-4 Individual 85	} " " " " "
Lines 5-6 Family No. 2 (sample No.12)	} Homozygous common allele
Family No. 2 (sample No. 13)	} " "
Line 7 Band stab individual 48	} " " Only used primer DS 867.R

To compare the sequencing from the reverse and forward primers, the sequencing results from the 3'UTR reverse primer (DS 867.R) were rotated and the complementary sequence was given in Table 7.2.

The results were similar to the band stab sequencing, except the sequencing was more accurate and precise, when compared to Widada et al.'s published sequence. Family No. 2 (13) did not sequence very well, so the results were not included in Table 7.2. The band stab individual 48 was sequenced from primer DS 867 (3'UTR reverse), as the template sequenced well last time in the forward direction in Table 7.1 and so it was repeated in Table 7.2, in the reverse direction.

The suspected polymorphism was at base 88 this time in Table 7.2. All the rare alleles were adenine again at this base. For the common alleles: sample 5 read a guanine, sample 6 - a thymine and sample 7 (the band stab of individual 48) - an adenine, yet last time it sequenced as a guanine. So out of 9 rare alleles sequenced, 9 out of 9 times it sequenced as adenine, which is different to Widada et al.'s guanine. 4 out of the 6 common alleles sequenced agreed with Widada et al.'s guanine at the polymorphic site. The other two samples read once an adenine and the other as a thymine, though the thymine sequence was quite bad (sample 6 - Table 7.2). So the base change causing the polymorphism still needed to be confirmed.

The variations from Widada's TPM2 sequence, mentioned in the band stab sequencing, were repeated for these samples:

There was a cytosine addition in Widada's sequence at base 91. 14 out of 15 independent sequencing samples of the rare and common alleles showed Widada et al. had an additional C. This may be explained, as in 1988 they would have used manual sequencing methods and it could have been fairly easy to read two C's in a row as three C's, as the bands would have been very close (Laing, 1994a).

11 out 15 independent sequencing samples showed that Widada et al. had a guanine deletion at base 97 in Table 7.2. However, there was no C substituted for a G at base 98 like in Table 7.1 and it was likely that Table 7.2 sequencing was more accurate.

14 out of 15 independent sequencing samples showed Widada et al. had an AG insertion after base 152 in Table 7.2. This was not shown in Widada's sequence, as otherwise all the bases from the other samples after this insertion would not be aligned with Widada's sequence and the sequence would have been more difficult to compare.

Therefore, there were four consistent base changes to Widada et al.'s published sequence in the 3'UTR from the sequencing results, which was similar to the sequencing in Table 7.1.

The sequence variation between the polymorphic alleles still needed confirmation, but it was suspected that the eleventh base in the 3'UTR was an adenine in the rare allele and a guanine in the common allele that caused the polymorphism, seen in the SSCP gels.

7.3.8 Restriction Digest of 259 bp TPM2 STS (8sk - 3'UTR)

Using the consistent TPM2 STS sequence defined by primers DS 870 and 867, a 259 base sequence was typed into a computer application package DNA Strider version 1.0. Two versions of the STS sequence were typed in, what was suspected for the common allele (Figure 7.9) and the rare allele (Figure 7.10). The application worked out most of the restriction sites for the two STSs. At base 83 (guanine) in Figure 7.9 (the common allele) there was a unique restriction site (*Bgl* I) present at the suspected site of the polymorphism that was not present in base 88 (adenine) for the rare allele, in Figure 7.10. This can be seen more clearly in Figure 7.11 showing a simplified restriction map of the common allele STS (a) and the rare allele STS (b). There is a unique restriction site (*Bgl* I) at base 83 seen in (a), but not in (b).

The sequence where the restriction enzyme *Bgl* I splices DNA is:

Bgl I restriction enzyme	GCCNNNN/NGGC
Suspected 259 bp TPM2 STS polymorphism (common allele)	GCCAGCG/TGGC
Suspected 259 bp TPM2 STS polymorphism (rare allele)	**A**CCAGCG/TGGC

This meant a restriction digest could be carried out on a PCR STS from different individuals. An STS from a homozygous individual for the rare allele would produce a single 259 bp fragment on a gel, as there is no restriction site for *Bgl* I. *Bgl* I would cut

an STS for a homozygous individual for the common allele into two fragments, a 88 bp and 171 bp fragment. The STS for a heterozygous individual would be cut into three fragments (88, 171 and 259 bp) as they have two alleles. One allele would not be cut by *Bgl* I (259 bp) and the other allele would be cut producing the two fragments (88 & 171 bp) (see Figure 7.12).

The restriction digest in Figure 7.13 showed that the TPM2 STS was polymorphic at base 88 and confirmed the sequencing results that it was the site for the polymorphism. Therefore, primers DS 870 (exon 8sk.F) and DS 867 (3'UTR.R) can now be used as unique markers in the human genome project for linkage analysis and entered on the CEPH linkage map. The polymorphism is quite useful, because the TPM2 gene has been localised to 9p13.1 and so the polymorphism when it is entered on the CEPH map can be correlated to the physical map.

FIGURE 7.9

Restriction Sites for the 259 bp TPM2 STS Sequence of the Common Allele.

The Common Allele has an extra Restriction Site (Bgl I) at Base 88.

DNA Strider 1.0 ### Sunday, 11 December 1994 10:48:30 PM

TPM2 Common Allele -> Restriction Map

DNA sequence 259 b.p. aagtctatgccc ... cttcatccttta linear

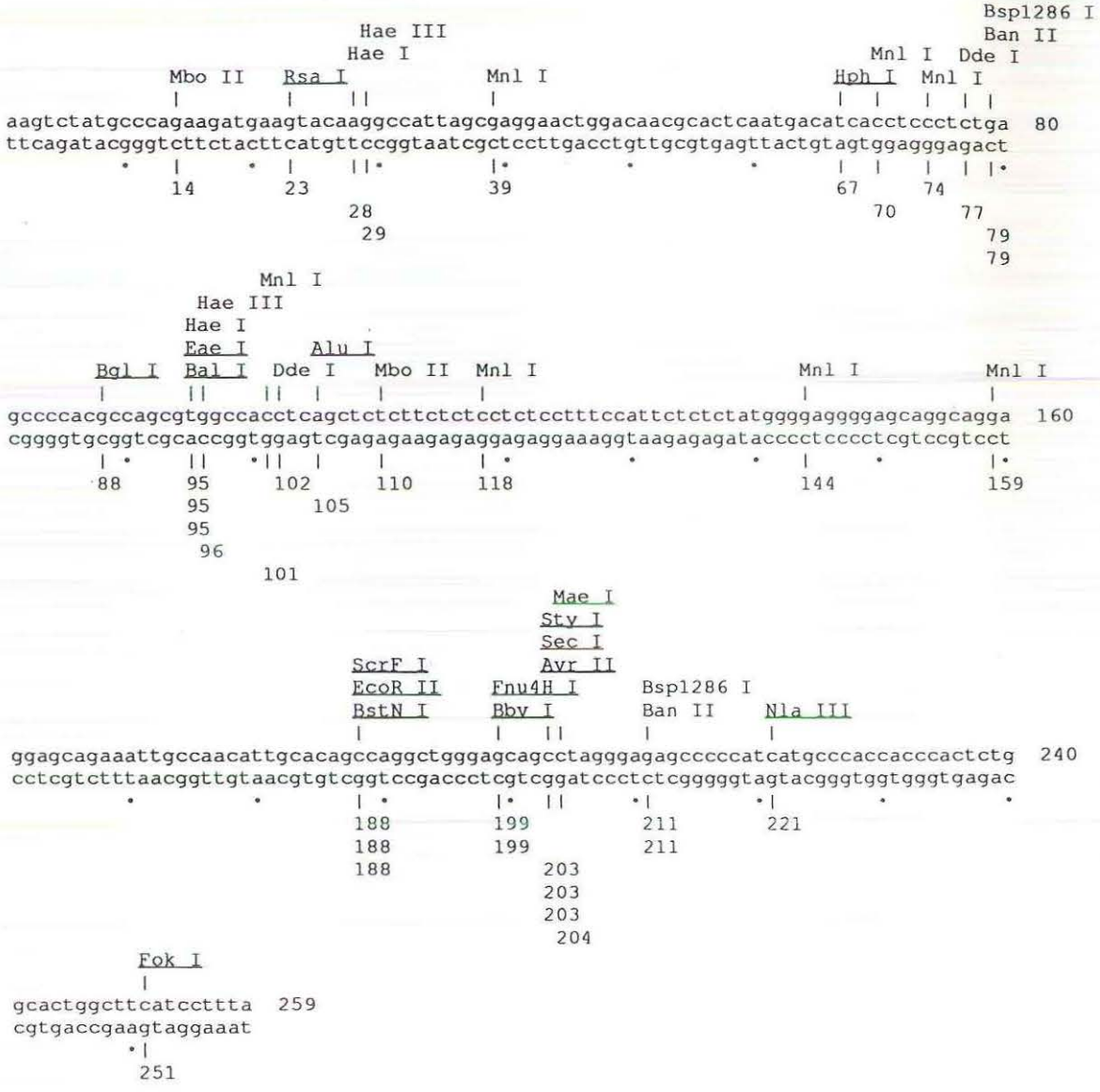


FIGURE 7.10

Restriction Sites for the 259 bp TPM2 STS Sequence of the Rare Allele

The Rare Allele does not have the Restriction Site (*Bgl* I) at Base 88.

DNA Strider 1.0 ### Sunday, 11 December 1994 10:50:15 PM

TPM2 Rare Allele -> Restriction Map

DNA sequence 259 b.p. aagtctatgccc ... cttcatccttta linear

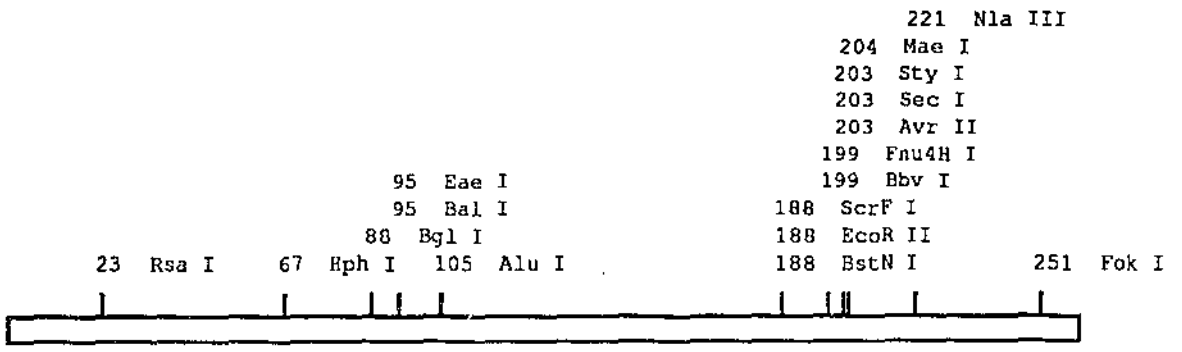
FIGURE 7.11

Graphic Map Showing the Restriction Sites of the 259 bp TPM2 STS

(A) The Common Allele has an extra Restriction Site (*Bgl* I) at Base 88.

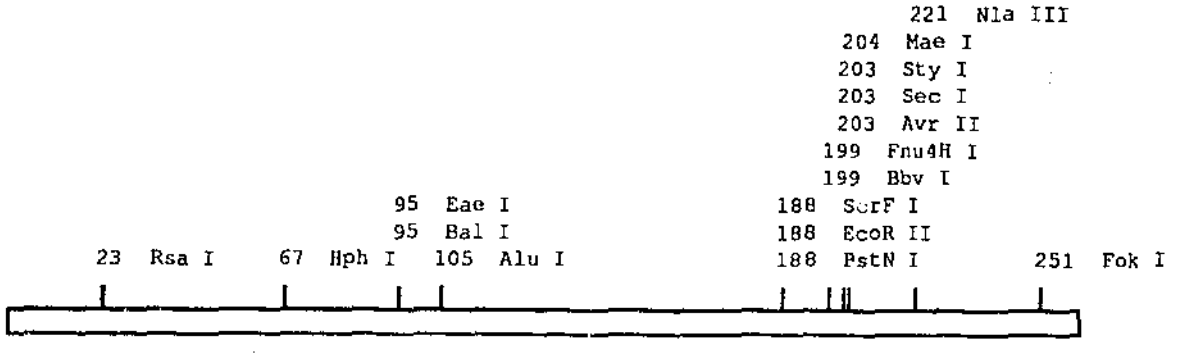
(B) The Rare Allele does not have the Restriction Site (*Bgl* I) at Base 88.

DNA sequence 259 b.p. aagtctatgccc ... cttcatccttta linear



TPM2 Common Allele 259 base pairs Unique Sites

DNA sequence 259 b.p. aagtctatgccc ... cttcatccttta linear



TPM2 Rare Allele 259 base pairs Unique Sites

FIGURE 7.12

Predicted Restriction Digest of 259 bp TPM2 STS (Exon 8sk - 3'UTR) Polymorphism using *Bgl* I

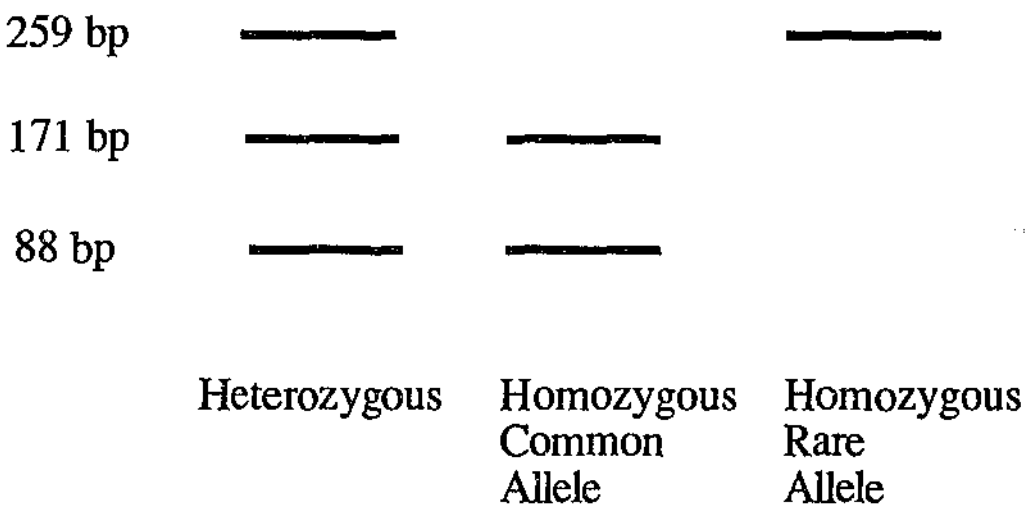

259 bp
171 bp
88 bp
Heterozygous
Homozygous Common Allele
Homozygous Rare Allele

FIGURE 7.13

Restriction Digest of 259 bp TPM2 STS (Exon 8sk - 3'UTR) Polymorphism using *Bgl* I

Lane 1	pGEM Marker
Lane 2	Heterozygous Individual for Allele
Lane 3	Homozygous Individual for Common Allele
Lane 4	Homozygous Individual for Rare Allele
Lane 5	pGEM Marker

pGEM

Heterozygous Allele

Homozygous Common Allele

Homozygous Rare Allele

pGEM

CHAPTER 8

GENERAL DISCUSSION

8.1 Localised Inherited Muscle Disorders

At present there are no known inherited muscle disorders linked to the 9p13 region (Klinger, 1992). The alpha-tropomyosin gene TPM1 has recently been localised to 15q22 (Eyre, et al., 1994) and has been shown to be mutated in some cases of familial hypertrophic cardiomyopathy (Thierfelder, et al., 1994). The alpha-tropomyosin gene TPM3 has been recently localised to 1q22-q23 (Wilton, et al., 1995) and has been shown to be mutated in a family with autosomal dominant nemaline myopathy (Laing, et al., 1994). Therefore, at present, TPM2 becomes a candidate disease causing gene for any unlinked inherited myopathies and muscle related disorders. Also, in the future inherited muscle disorders may be linked to chromosome 9p13 and, in this case, TPM2 would become a candidate disease causing gene.

8.2 Determining if the 259 bp TPM2 STS is a Pseudogene

It is extremely unlikely that the 259 bp TPM2 STS is a pseudogene as the STS was sequenced and it had a muscle specific exon 8sk, which will not be found in processed pseudogenes (2.1.2.2.). However, this STS could be excluded as a pseudogene, if it were radiolabelled and then the STS hybridised to a genomic blot cut with a restriction enzyme, such as *Taq* I, that does not cut the STS. If the autorad revealed only a single band, then the STS would be most likely hybridising to the TPM2 gene alone and not to pseudogenes. However, because a processed pseudogene has a similar sequence to the real gene, it can be cut by the restriction enzyme, and travel in the gel to the same location on the Southern blot as the real gene. So when the probe hybridises as a single band it could be hybridising to the real gene, or the pseudogene. By hybridising the STS to a few genomic blots, cut with different restriction enzymes, then if this probe hybridises as

a single band to all the genomic blots, one can be confident the STS is belongs to the real TPM2 gene. This is because it is extremely unlikely that the pseudogene would always be cut at the same points as the real gene, with the different restriction enzymes. The greater the number of different genomic blots, cut with different restriction enzymes, that are used, the greater the certainty that this is the TPM2 gene.

8.3 Long Range PCR STSs for use in FISH

The methods of isolating genomic clones for use in FISH have almost been superseded with the new technique of long range PCR. Since 1992 researchers have increased the conventional range of PCR from typically 5 kb to now 5 kb to 40 kb, with averages of 5 - 15 kb in genomic DNA (Cheng, et al., 1994; Kainz, et al., 1992; Ponce & Micol, 1992). The key to long range PCR is the new DNA polymerase, *UlTma*™ DNA polymerase which has an inherent 3' - 5' exonuclease ("proofreading") activity which not only increases the length of PCR fragments produced, but also the accuracy of the amplified copies. Perkin Elmer have produced a PCR kit in 1994 that can perform long range PCR (Perkin-Elmer, 1994).

Producing long PCR fragments increases the amount of specific genomic DNA comparable to genomic clones (8 - 21 kb) that can be used in FISH. This, therefore, greatly reduces the time and effort in localising genes by isolating a genomic clone. The only problem is that the technique is new and many of the PCR parameters still need to be optimised. Also there is no guarantee that a long range PCR product will work in FISH (Wilton, 1994). Likewise, however, there is no guarantee that isolating a genomic clone for use in FISH will be successful.

8.4 Linkage of the TPM2 Polymorphism

Linkage of a polymorphism is performed using known markers. These are usually polymorphic regions of DNA like the polymorphism for TPM2 described in chapter 7. The 259 bp TPM2 STS polymorphism is called a restriction fragment length polymorphism (RFLP), because the restriction enzymes produce different sized fragments in polymorphic STSs (Gelehrter & Collins, 1990).

Another more informative class of marker in the genome are microsatellites. Microsatellites are tandem repeats of base sequences, usually less than 100 bp in total length and occur on average approximately every 6000 bp in the human genome. Since humans have $3x10^9$ bp in the genome, then there are about $5x10^9$ microsatellites, so every gene should have a microsatellite near it (Cooper, 1994).

The function of microsatellites is not well understood, but one theory is that they are common locations for recombinations (Cooper, 1994). Of the microsatellite repeat sequences, 76% of them in decreasing frequency are:

A, AC, AAAN, AAN, or AG

N = any nucleotide A G C or T

(Cooper, 1994)

Microsatellites are inherited in a strict Mendelian pattern, except for the rare occasions when the number of repeats alters from one generation to the next by mutation. Different alleles may vary in the repeat number, for example, there may be an $(AC)_n$ repeat on a chromosome at a known locus. A mother may have 4 and 9 tandem repeats of the sequence on the two different alleles at the locus and the father 6 and 3 repeats. Therefore a child of theirs can only have one tandem repeat from either allele of the parent, with the following possible combinations - 9,6; 9,3; 4,6; and 4,3 unless there is a mutation. These microsatellites would be included in an PCR STS that can be identified by acrylamide gel

electrophoresis, to determine the segregation of alleles for different individuals in a pedigree. In this way they are more informative than a RFLP, as there are more combinations of microsatellite alleles at a locus, in a pedigree, than a single RFLP (Sheffield & Stone, 1994).

Another marker is minisatellites or variable number of tandem repeats (VNTRs) which were discovered before microsatellites. VNTRs are less common and less evenly distributed through the genome, so they are not quite as useful as microsatellites. VNTRs are bigger than microsatellites and their repeating unit is 30 to 35 bp in length and have a variable sequence but contain a repeating core sequence 10 to 15 bp in length. Minisatellite repeats range in size from 200 bp up to several thousand bp and tend to occur in greater numbers toward the telomeric ends of chromosomes. Because VNTRs are much bigger than microsatellites they are harder to amplify using PCR. They are separated on agarose (not acrylamide gels) or are probed on Southern blots (Cooper, 1994).

8.5 Linkage Results and CEPH Mapping

Helen Eyre and Dr John Mulley at the Department of Cytogenetics and Molecular Genetics at Adelaide Children's Hospital, have linked the TPM2 polymorphism in CEPH families, in comparison with known markers in the region 9p21-13. This confirms the FISH localisation to 9p13 as well as the somatic cell hybrid localisation to chromosome 9.

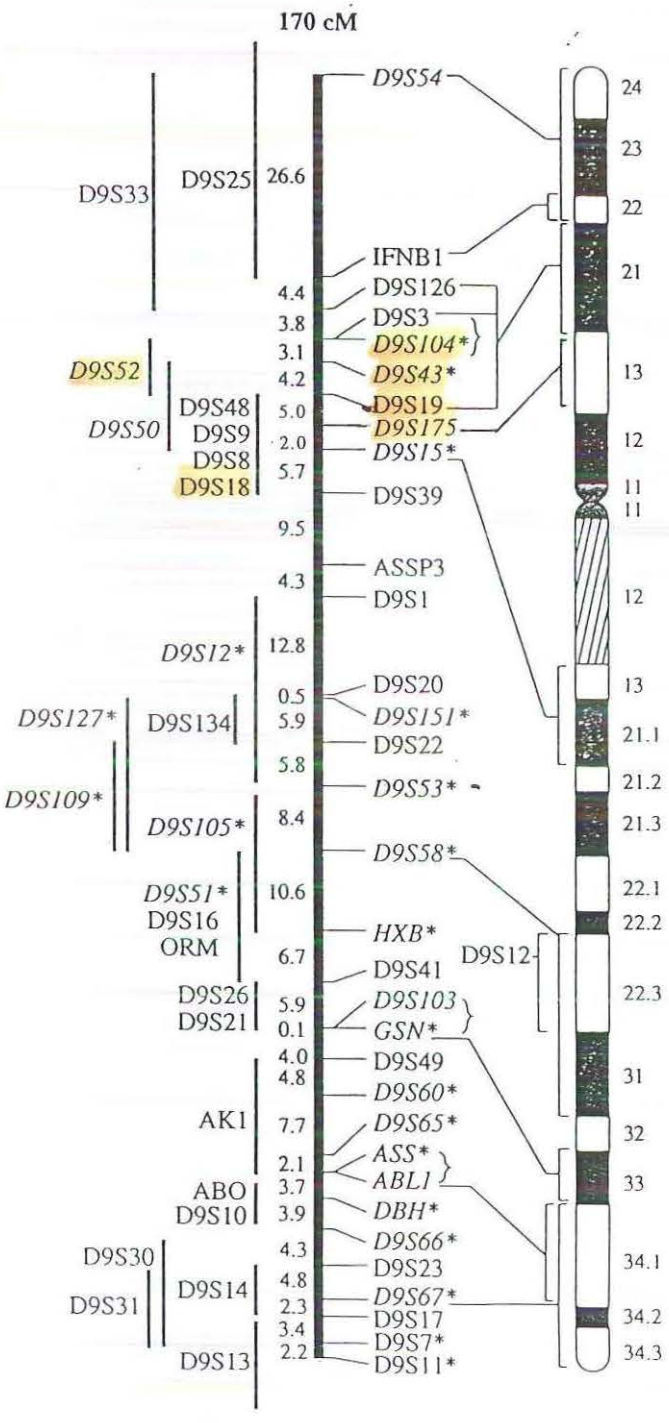
The distance apart of two loci, is defined as in linkage terms as the Kosambi mapping function, which is measured in centiMorgans (cM). If two loci are 1 cM apart, there is an approximate, average probability of 0.01 of recombination taking place between these loci. It is difficult to be more precise than this because recombination frequency varies throughout the genome and between the sexes (Gelehrter & Collins, 1990).

FIGURE 8.1

Genetic linkage map of sex-averaged chromosome 9, showing CEPH polymorphic markers. An asterix (*) next to a marker indicates loci with heterozygosities of at least 70%. Microsatellite markers assayed by PCR are shown in italics. The graphic chromosomal idiogram depicts the Giemsa staining pattern. The D9S numbers to the right of the sex averaged map that point to the chromosomal idiogram, indicate those markers that have been cytogenetically mapped.

(NIH/CEPH Collaborative Mapping Group, 1992)

9


170 cM
D9S54
24
23
D9S33
D9S25
26.6
22
IFNB1
21
4.4
D9S126
3.8
D9S3
3.1
D9S104*
D9S52
4.2
D9S43*
13
D9S48
5.0
D9S19
D9S50
D9S9
2.0
D9S175
12
D9S8
D9S15*
D9S18
5.7
11
D9S39
11
9.5
ASSP3
4.3
D9S1
12
D9S12*
12.8
D9S20
13
0.5
D9S127*
D9S134
5.9
D9S151*
21.1
D9S22
5.8
D9S53*
21.2
D9S109*
8.4
D9S105*
21.3
D9S58*
D9S51*
10.6
22.1
D9S16
22.2
HXB*
ORM
6.7
D9S41
D9S12
D9S26
5.9
D9S103
22.3
D9S21
0.1
GSN*
4.0
D9S49
4.8
D9S60*
31
AK1
7.7
D9S65*
32
2.1
ASS*
ABL1
3.7
33
ABO
D9S10
3.9
DBH*
D9S66*
4.3
D9S30
D9S23
34.1
4.8
D9S14
D9S67*
D9S31
2.3
D9S17
34.2
3.4
D9S7*
D9S13
2.2
D9S11*
34.3

The most likely location for the TPM2 SSCP was between markers D9S19 (a Taq I RFLP) and D9S175 (an $(AC)_n$ microsatellite repeat) (the highlighted markers in Figure 8.1, were those found to be closely linked to the TPM2 polymorphism). The DS numbers refer to the CEPH notation system. The 9 after the D refers to chromosome 9 and the numbers after the S are the order in which the polymorphic markers were found. (Klinger, 1992).

TABLE 8.1

Markers and their Recombination Frequency with the TPM2 Polymorphism

Locus Symbol	Recombination Frequency with TPM2 Polymorphism	cM
D9S104	0.02	19.7
D9S52	0.00	21.3
D9S43	0.00	22.1
D9S18	0.08	25.2
D9S19	0.00	33.0
TPM2 SSCP		
D9S175	0.08	47.2

The low recombination values between the markers in Table 8.1 and the TPM2 SSCP indicate that they are closely linked to TPM2. However, with a heterozygosity of 0.16, the TPM2 polymorphism is relatively uninformative and the chances of recombination between the TPM2 SSCP and these markers in the CEPH families is quite low. This means that caution must be exercised in interpreting these results. A more informative marker closely linked to TPM2 is required to confirm the localisation of TPM2 between D9S19 and D9S175.

8.6 Finding a Microsatellite Close to the TPM2 Gene

The most informative markers used in gene mapping projects today are microsatellites. Had time permitted, the next stage of this project would have been the discovery of a microsatellite closely linked to TPM2. There are two ways in which the identification of such a microsatellite could have been achieved.

Firstly, from a yeast artificial chromosome or YAC contig covering the presumed D9S19 - D9S175 linkage region of TPM2, a YAC clone(s) would have been isolated that contained a TPM2 STS. Microsatellites would then be sought in any TPM2 positive YAC(s).

YACs are cloning vectors that replicate as chromosomes in yeast cells and can accommodate human DNA inserts as large as 1 million base pairs. These large inserts are extremely useful for attaining long-range continuity in contig or overlapping maps and are therefore useful in large-scale mapping of the human genome (Cooper, 1994).

A YAC contig covering the 9p13 region would have been identified using the MOSAIC database program. The YAC(s) in the 9p13 contig containing all (or part) of the TPM2 gene would then be identified by PCR assay using the YAC(s) as target DNA for the TPM2 STS primers. Those YAC clones that produced a positive result must contain a segment of the TPM2 gene. An important control for the YAC TPM2 STS PCR would be the yeast DNA of *S. cervisae* and the TPM2 STS primers. *S. cervisae* is the yeast host strain for the 9p13 YAC contig. Homology between the TPM2 STS primers and *S. cervisae* DNA does not occur (Genebank), nevertheless a negative yeast DNA control for the PCR amplification is required (Laing, 1994a).

Positive TPM2 PCR STS assay isolated clones, would then be screened for di, tri, tetra and penta nucleotide tandem repeat sequences (or microsatellites), especially the most common $(CA)_n$ repeat most typical of microsatellite polymorphisms. This would have

been achieved by digesting DNA from isolated YAC clones specific to TPM2 STSs with restriction enzymes and then identifying those fragments which anneal to ^{32}P labelled eg. $(GT)_{10}$ on a Southern blot. Once localised, these $(CA)_n$ containing fragments would be subcloned into a selected plasmid, using a modification to the technique described by (Feener, et al., 1991). The sequence of the plasmid either side of the insert is known and primers have been designed so the insert can be PCR amplified and then sequenced. An insert less than 1000 bp would be ideal, as the maximum sequencing range from each primer on either side of the insert is approximately 500 bp. If the insert is much larger than 1000 bp, then the fragment would be cut again with another restriction enzyme and reprobed on a Southern blot and then subcloned and sequenced. Alternatively new primers could be designed from the information obtained at the end of one sequence to continue the sequencing of the insert until the microsatellite is sequenced. Primers would then be designed on either side of the microsatellite to produce an STS that includes the microsatellite (Laing, 1994a; Sambrook, et al., 1989).

A random population can be screened using these primers to determine the allelic frequency of these microsatellite polymorphisms, as in 7.2.7. Once a suitable panel of microsatellites had been identified, they would have been screened against the CEPH panel of families in collaboration with Department of Cytogenetics and Molecular Genetics at Adelaide Children's Hospital to localise the polymorphisms position on the CEPH map (Laing, 1994a).

Similarly, somatic cell hybrids that contain parts of chromosome 9p13 can be isolated using TPM2 STSs and the methods repeated, as for YACs to isolate a microsatellite repeat that can be localised in the CEPH map, that would also have a physical location to 9p13 (Laing, 1994a).

8.7 Conclusion

The somatic cell hybrid and FISH localisation of human skeletal tropomyosin (TPM2) to 9p13 paper has been accepted by the *Cytogenetics and Cell Genetics Journal*. (see Appendix 3). The detection of the *Bgl* I RFLP in the 3'UTR of TPM2, and linkage of this polymorphism will be submitted to *Genomics* and entered on the CEPH map.

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APPENDIX 1

TROPOMYOSIN GENE

SEQUENCES

TPM1 - McLeod and Gooding, (1988).

TPM2 - B tropomyosin - Widada et al., (1988).

TPM3 - Reinach and McLeod, (1986).

TPM4 - MacLeod, Talbot, Smillie, Houlker - J Mol Biol 194:1-10, (1987).

cccgctccgtcctcctcgcctgccaccg

DS1095 TPM1-Ex1-F

ccgcgcgctcgcccc**gccgctcctgctgcag**ccccaggcccctcgccgccgccacc

DS1083 TPM2-Ex1-F

gtgcacccagtccgctcacccagcccagtccgtccggtcctcaccgcctgccggccggcccacccccccaccgcaggcc

DS886 TPM3(14)

ttgccccagtctccagttctccagtgttcacaggtgagcctaccaacagccactgctc

Exon Isk

atg

atggacgccatcaagaagaagatgcagatgctgaagctcgacaaggagaacgccttggatcgagctgagcaggcggag

DS840 TPM2.1skF

*atggacgccatcaagaagaagatgcagatgctgaagctggacaaggaga**acgccatcgaccgcgccgagcaggccgaa***

atggaggccatcaagaaaaagatgcagatgctgaagttagacaaggagaatgctctggatcgggctgagcaagctgaa

DS1094 TPM1-Ex1-R

gccgacaagaaggcggc**ggaagacaggagcaagcag**

DS1082 TPM2-Ex1-R

*gccgacaagaagcaagc**tgaggaccgctgcaagcag***

DS1018 TPM3-2002

gctgagcagaagcagg**cagaagaaagaagtaaacag**

Exon IIsk

ctggaagatgagctggtgtcactgcaaaagaaactcaagggc

ctggaggaggagcagcaggccctccagaagaagctgaagggg

ctggaggatgagctggcagccatgcagaagaagctgaaaggg

accgaagatgaactggacaaatactctgaggctctcaaagatgcccaggagaagctggagctggcagagaaaaaggcc

DS841 TPM2.IIskR

acagaggatgaggtggaaaagtattctgaatccgtgaaggaggcccaggagaaactggagcaggccgagaagaaggcc

acagaggatgagctggacaagtattctgaagctttgaaggatgcccaggagaagctggaactggcagagaagaaggct

accgat

actgat

gctgat

Exon II

gctgaagccgacgtagcttctctgaacagacgcatccagctggttgaggaagagttggatcgtgcccaggag

gctgaggcagatgtggcctccctgaaccgccgcattcagctggttgaggaggagctggaccgggcccaggag

gctgaggctgaggtggcctccttgaaccgtaggatccagctggttgaagaagagctggaccgtgctcaggag

DS1372-

gctgaaggtgatgtggccgccctcaaccgacgcatccagctcgttgaggaggagttggacagggctcaggaa

cgtctggcaacagctttgcagaagctggaggaagctgagaaggcagcagatgagagtgagag

cgcctggctacagccctgcagaagctggaggaggccgagaaggcggctgatgagagcgagag

cgcctggccactgccctgcaaaagctggaagaagctgaaaaagctgctgatgagagtgagag

TPM4IIF

cgactggccacggccctgcagaagctggaggaggcagaaaaagctgcagatgagagtgagag

Exon III

aggcatgaaagtcatt
aggaatgaaggtcatc
aggtatgaaggttatt
aggaatgaaggtgata

gagagtcgagcccaaaaagatgaagaaaaaatggaaattcaggagatccaactgaaagaggccaagcacattgctgaa
gaaaaccgggccatgaaggatgaggagaagatggaactgcaggagatgcagctgaaggaggccaagcacatcgctgag
gaaaaccgggccttaaaagatgaagaaaagatggaactccaggaaatccaactcaaagaagctaagcacattgcagaa

gaaaaccgggccatgaaggatgaggagaagatggagattcaggagatgcagctcaaagaggccaagcacattgcggaa

gatgccgaccgcaaatacgaagag
gattcagaccgcaaatatgaagag
gaggcagataggaagtatgaagag
DS1373 - TPM4IIIR
gaggctgaccgcaaatacgaggag

Exon IV

gtggcccgtaagctggtcatcattgagagcgacctggaacgtgcagaggagcgg
gtggccaggaagctggtgatcctggaaggagagctggagcgctcggaggagagg
gtggctcgtaagttggtgatcattgaaggagacttggaacgcacagaggaacga
DS1052 TPM4.4F
gtagctcgtaagctggtcatcc**tggagggtgagctggagagggcagaggagcgt**

gctgagctctcagaagg
gctgaggtggccgagag
gctgagctggcagagtc
gcggaggtgtctgaact

Exon Vsk

g g TPM1a=rat
caaatgtgccgagcttgaagaa**gaattgaaaactgtgacg**aacaacttgaagtcactggag
DS752 TPM2.VF
taaatgtggggacctagaggaggagctgaaaattgttaccaacaacttcaaatccctggag
taagtgttctgagctggaggaggagctgaagaatgtcaccaacaacctcaagtctcttgag

gctcaggctgagaag
gcccaggcggacaag
gctcaggcggagaag

Exon VI

tactcgcagaaggaagacagatatgaggaagagatcaaggtcctttccgacaagctgaaggag
DS869 TPM2.6F
tattccaccaaagaagataaa**tatgaagaggagatcaaactgttggaggagaagctgaaggag
tactctcaaaaagaagataaatatgaggaagaaatcaggattcttactgataaactcaaggag
DS754 - TPM4a
tattctgaaaaggaggacaaatatgaagaagaaattaaacttctgtctgacaaactgaaagag

Exon VII

TPM1b=rat
gctgagactcgggctgagtttgcggagaggtcagtaactaaattg**gagaaaagcattgatgacttag**aag
DS868 TPM2.7F DS753 TPM2.7R
gctgagacccgagcagagtttgccgagaggtctgtggcaaagttggagaaaaccatcgatgacctagaag
gcagagacccgtgctgagtttgctgagagatcggtagccaagctggaaaagacaattgatgacctggaag

DS755 TPM4b

gctgagacccgtgctgaatttgcagagagaacggttgcaaaactggaaaagacaattgatgacctggaag

Exon VIIIsk

acgagctg
atgaagtc
atgagctc

tacgctcagaaactgaagtacaaagccatcagcgaggagctggaccacgctctcaacgatatgacttccata
DS870 TPM2.8F DS866 TPM2.8R
tatgcccagaagatg*aagtacaaggccattagcgaggaactggacaacgcac****tcaatgacatcacctccctc***
tatgcccagaaactgaagtacaaggccattagcgaggagctggaccacgccctcaatgacatgacctctata

3' untranslated

taagtt
tgagcc
taatta

tctttgcttcacttctcccaagactccctcgtcgagctggatgtcccacctctctgagctctgcatttgtctattctc
ccacgcccagcgtgccacctcagctctcttctctcctctcctttccattctctctatggggaggggagagcaggcagg
tcaccgtttctgctctgttctggatctgccccctttactcctcggggaacccaaggccccactctcgctctggattcc

cagctgaccctggttctctctcttagcatcctgccttagagccaggcacacactgtgctttctattgtacagaagctc
aggagcagaaattgccaacattgcacagccaggctgggagcagcctagggagagcccccatcatgcccaccacccact
atttgggtcagcctggctggtccccaaggcattaggatgggggagcaaaaagcaacttatgtattttcttccacccccc

ttcgtttcagtgtcaaataaacactgtgtaagctaaaaaaa
DS867 TPM2.3'UTR
ctggcactggcttcatcctttacctatcccctccaccctcctttgcttgcttaataaattctgaacttggaaaaaaa
accccaaattaaaatgttaagctgctggaaaaaaaaaa

aa

APPENDIX 2

BUFFERS AND SOLUTIONS

GENERAL SOLUTIONS

% Agarose

Weight agarose per volume in 1X TAE

ie. 1% Agarose gel = 1g agarose in 100 mL 1X TAE

0.5M EDTA (pH 8.0)

Add 186.1 g of disodium ethylenediaminetetra-acetate·$2H_2O$ to 800 mL of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20g of NaOH pellets). The disodium salt of EDTA does not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH. Dispense into aliquots and sterilise by autoclaving (Sambrook, et al., 1989).

10% SDS (Sodium dodecyl sulfate or sodium lauryl sulfate

Dissolve 100 g of electrophoresis-grade SDS in 900 mL of H_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 L with H_2O. A mask was worn weighing out the SDS as the fine crystals dispersed easily (Sambrook, et al., 1989).

20X SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 L with H_2O. Sterilise by autoclaving (Sambrook, et al., 1989).

STE (Sodium Chloride, Tris, EDTA)

0.1 M NaCl

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0) (Sambrook, et al., 1989)

TE (Tris EDTA Buffer)

TE is used as a buffer for DNA. DNA being a weak acid will undergo autocatalysis in the absence of a buffering agent (Sambrook, et al., 1989).

1M Tris

Dissolve 121.1 g of Tris base in 800 mL of H_2O. Adjust the pH to the desired value by adding concentrated HCl. Allow the solution to cool to room temperature before making the final pH adjustments, as the pH of Tris is temperature dependent. Adjust the volume to 1 L with H_2O. Sterilise by autoclaving (Sambrook, et al., 1989).

5X Tth Buffer

335 mM Tris HCl (pH8.8) at 25°C

83 mM $(NH_4)_2 SO_4$

1 mM each dNTPs

10 mM $MgCl_2$,

1 mg gelatin/ml

2.25% Triton (X-100)

(ANRI, 1994)

ETHIDIUM BROMIDE

STOCK CONCENTRATION	STAINING CONCENTRATION
10 mg/mL (1g Ethidium Bromide in 100mL H_2O	0.5 µg/mL

ELECTROPHORESIS BUFFERS

BUFFER	WORKING SOLUTION	CONCENTRATED STOCK SOLUTION (per litre)
Tris-acetate (TAE)	1X: 0.04 M Tris-acetate 0.001 M EDTA	50X: 242 g Tris base 57.1 mL glacial acetic acid
Tris-borate (TBE)	1X: 0.09 M Tri-borate 0.002 M EDTA	10X : 108 g Tris base 27.5 g boric acid 20 mL 0.5 M EDTA (pH 8.0) use double distilled water as 10X TBE otherwise precipitates

(Sambrook, et al., 1989)

GEL LOADING BUFFERS

BUFFER TYPE	BUFFER	STORAGE TEMPERATURE
Agarose Buffer		
Ficoll (6X)	0.25% bromophenol blue	room temperature
	0.25% xylene cyanol FF	
	15% Ficoll (Type 400;	
	Pharmacia in water	
SSCP Buffer		
Formamide (2x)	1 mL deionised formamide	4°C
	1 EDTA (pH 8.0)	
	0.025% bromophenol blue	
	0.025% xylene cyanol FF	

(ANRI, 1994)

BACTERIAL MEDIA AND REQUIRED SOLUTIONS

1.5% bottom agar

Add agar (Difco Laboratories) weight to volume of 2X YT and made to 10 mM $MgSO_4$ and 0.2% maltose.

For example to make 1 L of bottom agar added:
15 g agar/L and autoclave to sterilise and cool to approximately 50°C. Usually some volume is lost so make to 980 mL with distilled water.
At 50°C add 10 mL 20% maltose and 10 mL 1 M $MgSO_4$. The solution was left to cool to around 50°C because at high temperatures the maltose caramelises.

(Sambrook, et al., 1989)

0.7% top agarose

Add agarose (Progen Industries) weight to volume of 2X YT and made to 10 mM $MgSO_4$ and 0.2% maltose.

For example to make 100 mL of top agarose added:
0.7 g agarose to 100 mL of 2X YT and boiled in microwave until dissolved.
Cooled to approximately 50°C and added 1 mL of 20% maltose and 1 mL 1 M $MgSO_4$.

(ANRI, 1994)

DNA denaturing solution

1.5 M	NaCl
0.5 M	NaOH

(Clontech, 1994)

10X Lambda dilution buffer

58.3 g 1.0 M NaCl

24.65 g 0.1 M $MgSO_4.7H_2O$

350.0 mL 1.0 M Tris-HCl (pH 7.5) (final concentration 0.35 M)

Added H_2O to a final volume of 1 L. Autoclaved and stored at 4°C (Clontech, 1994).

1X Lambda dilution buffer

100 mL 10X Lambda dilution buffer

5 mL 2% Gelatin

Added H_2O to a final volume of 1 L. Autoclaved and stored at 4°C (Clontech, 1994).

100 mL 20% Maltose

20 g maltose in 80 mL H_2O. Filter sterilised (ANRI, 1994).

100 mL 1 M $MgSO_4$

Added 24.65 g $MgSO_4$ with H_2O to make 100 mL. Filter sterilised using 0.22 µm pore filter unit (Millipore - Millex - GS) and 50 mL syringe (Terumo) (ANRI, 1994).

Phenol:Chloroform

Mix equal amounts of equilibrated phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris-HCl (pH 7.5). Store the equilibrated mixture under an equal volume of 0.01 M Tris-HCl (pH 7.5) at 4°C in dark glass bottles.
Phenol must be equilibrated to a pH > 7.8 because DNA will partition into the organic phase at acidic pH (Sambrook, et al., 1989).

Chloroform:Isoamyl Alcohol (24:1)

A mixture of chloroform is mixed with isoamyl alcohol in the ratio of 24:1 respectively. The mixture is used to remove proteins from nucleic acids. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction from the SDS. The chloroform:isoamyl alcohol mixture is stored under 0.1 M Tris-HCl (pH 8.0) in a dark bottle at 4°C for periods of up to one month (Sambrook, et al., 1989).

Tris neutralising solution

1.5 M	NaCl
0.5 M	Tris-HCl (pH 7.5)

(Clontech, 1994)

2X YT Medium

Per litre:

To 900 mL of MilliQ water (deionised water)

Bacto-trypone	16g
Bacto-yeast extract	10g
NaCl	5g

Mixed until solutes had dissolved and made up to 1 L with H_2O

Sterilised by liquid autoclave.

(Sambrook, et al., 1989)

POLYACRYLAMIDE GEL ELECTROPHORESIS FOR SSCP

GELS AND SOLUTIONS USED

40% Acrylamide Stock (39:1 Acylalmide:Bisacrylamide)

Wearing a face mask, 390 g acyrlamide and 10 g bisacylamide were dissolved in 600 mL of MilliQ water. The solution was deionised by adding two dessert spoonfuls of Mixed Bead Resin (AG 501-X8 (D) Resin, 20-50 mesh, Bio-Rad) and stirring for 30 min. The solution was filtered using 305 mm diameter Postlip Paper (Hollingsworth & Vose Co. Ltd). The bottle was wrapped in foil and kept at 4°C (Sambrook, et al., 1989).

6% or 12% Nondenaturing SSCP Gel

To make a 12% nondenaturing SSCP gel, 300 mL of the 40% acrylamide stock was dissolved in 600 mL of MilliQ water and 100 mL of 10X TBE was added.

To make a 6% nondenaturing SSCP gel, 150 mL of the 40% acrylamide stock was dissolved in 750 mL of MilliQ water and 100 mL of 10X TBE was added.

Ammonium persulphate

Ammonium persulphate was made up at 25% weight/volume in MilliQ water and stored at 4°C, usually 2.5 g in 10 mL of MilliQ water (ANRI, 1994).

Fixative (10% Methanol and 10% Acetic Acid)

Usually make 1L by adding 100 mL of glacial acetic acid and 100 mL of methanol to 800 mL of MilliQ water in a fumehood cupboard (ANRI, 1994).

APPENDIX 3

TPM2 FISH LOCALISATION TO 9p13

ACCEPTED FOR PUBLICATION BY CYTOGENETICS AND CELL GENETICS

Assignment of the human beta tropomyosin gene (TPM2) to band 9p13 by fluorescence in situ hybridisation

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Running title: Localisation of TPM2 to 9p13

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Abstract. A sequence tagged site (STS) was developed for the human beta tropomyosin gene (TPM2). The STS was used to amplify DNA from somatic cell hybrids to localise TPM2 to human chromosome 9. Genomic clones isolated with the STS product were in turn used in fluorescence in situ hybridisation to metaphase chromosome spreads to further localise TPM2 to 9p13.

Each muscle-specific gene should be associated with an inherited muscle disease, unless mutations do not occur in that gene or all mutations of that gene are lethal. Precise mapping of muscle genes therefore becomes important in relation to mapping muscle diseases (Eyre et al., 1993).

Tropomyosin is one of the components of the thin filaments of muscle, binding to actin, and, together with troponin, regulating contraction in a calcium-dependent manner (Cho et al.,1990). There are at least four distinct tropomyosin genes in vertebrates and each may encode six different isoforms of tropomyosin by alternate splicing (Novy et al., 1993; MacLeod et al., 1988). The alpha-tropomyosin gene TPM1 has recently been localised to 15q22 (Eyre et al., 1994) and has been shown to be mutated in some cases of familial hypertrophic cardiomyopathy (Thierfelder et al., 1994). The alpha-tropomyosin gene TPM3 has been recently localised to 1q22-q23 (Wilton et al., 1994) and has been shown to be mutated in a family with autosomal dominant nemaline myopathy (manuscript submitted).

We describe the mapping of the human beta tropomyosin gene TPM2 (Widada et al., 1988) to 9p13 by development of a sequence tagged site (STS) (Olson et al., 1989), testing of a somatic cell hybrid panel, and fluorescence in situ hybridization (FISH) (Callen et al., 1992).

Materials and Methods

STS for the TPM2 gene.

The cDNA sequence of the skeletal muscle transcript of the TPM2 gene (Widada, et al., 1988) was used along with the known conserved exon pattern of the tropomyosin genes (Ruiz-Opazo et al., 1987; Clayton et al., 1988) to design primers for amplification which would amplify across intronic sequences. The primers were chosen such that at least one was located in a skeletal muscle specific exon (Clayton, et al., 1988; Ruiz-Opazo & Nadal-Ginard, 1987; Widada, et al., 1988) to avoid amplifying pseudogene sequences. The primers eventually used were in the muscle specific exon VIIIsk (TPM2.8R: 5'-AGTTACTGTAGTGGAGGGAG-3') and the common exon VI (TPM2.6F: 5'-ATTCCACCAAAGAAGATAAA-3') (Widada, et al., 1988). These primers amplified an STS product of approximately 1.5 kb long. The amplification conditions for a reaction volume of 25µl were: 5µl of 5X buffer (335mM Tris HCl [pH8.8] at 25°C; 83mM $(NH_4)_2$ SO_4, 1 mM dNTPs, 10 mM $MgCl_2$, 1 mg gelatin/ml, and 2.25% Triton X-100), 50 ng target DNA and 0.5 Unit of Tth polymerase (Biotech International). Thermal cycling conditions were: 35 cycles of 94°C for 30 secs, 58°C for 6 mins, with an initial denaturing period at 94°C for 4.5 mins. The 1.5 kb product was sequenced using an ABI 373A DNA sequencer tc confirm that the STS product did indeed contain parts of the TPM2 gene.

Isolation of genomic clones

The genomic library was constructed in λEMBL3 SP6/T7 (Clonetech) following a partial BamH1 digestion. The library was plated out at 6-fold redundancy and duplicate lifts made using Hybond N+ colony/plaque screening membranes (Amersham). The probe used was the 1.5 kb PCR amplified STS of the TPM2 gene which was radiolabelled with α P^{32} dCTP (Bresatec) using a random-prime kit (Promega). The duplicate lifts were hybridised with probe at 42°C for 16 hours in a hybridisation solution of 50% formamide, 10% dextran-sulphate, 1M NaCl, 50mM Tris-HCl pH7.5, 0.1 mg/ml herring sperm DNA. Lifts were washed in 0.1% SDS and 0.1x SSC at 65°C for 30 minutes. Autoradiography was carried out with Dupont Cronex 4 X-ray film with Quanta

III autoradiography screens at -80°C overnight. Plaques showing up in duplicate on the autoradiographs were considered positive. Confirmation of the TPM2 sequence in the genomic clones was ensured by STS PCR assay and sequencing.

Pure clones were then amplified by the plate lysate method (Sambrook et al., 1989). Phage were eluted in Lambda dilution buffer (100mM NaCl, 10 mM $MgSO_4.7H_20$, 100 mM Tris pH 7.5, 0.01% gelatin solution) at 4°C overnight. Eluates were collected and phage were purified on caesium chloride gradients (Sambrook, et al., 1989) and DNA extracted. DNA integrity was checked by running each sample on a 0.8% agarose gel at 100 volts for 30 minutes and staining with ethidium-bromide. Extracted DNA was confirmed to contain the TPM2 gene by STS PCR and sequencing. Whole clones were then used for in situ hybridisation.

In situ hybridisation

The probes were nick-translated with biotin-14-dATP and each was hybridized in situ at a final concentration of 20 ng/µl to metaphases from two normal males. The fluorescence in situ hybridization (FISH) method was modified from that previously described (Callen et al., 1990) in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosome identification). Images of metaphase preparations were captured by a CCD camera and computer enhanced.

Cell lines

A panel of rodent-human somatic cell hybrids was obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository [NIGMS/mapping panel #2]. The construction of the mouse-human hybrid cell lines was described by Dubois and Naylor (1992). PCR amplification of the STS was performed with 50 ng of hybrid DNA and the primers TPM2.6F and TPM2.8R as above.

Results and discussion

Somatic cell hybrid panel

The TPM2 STS gave an amplification product from only the control human DNA and the somatic cell hybrid GM/NA10611 (Dubois et al., 1992) (data not shown). Only chromosome 9 was unique to this cell line indicating that TPM2 is on human chromosome 9.

FISH

The STS PCR product was used as a hybridization probe to the genomic library which has an average insert size of 15 kb. Four positive plaques were picked and respread at lower density enabling individual plaques to be isolated. The STS primer set was used to assay selected pure plaques. Two of those which gave positive STS signals, TPM2.2 and TPM2.4 , were further propagated.

TPM2.2 and TPM2.4 were then used for FISH to metaphase spreads from three normal males. Twenty-five metaphases from the first normal male were examined for fluorescent signal with TPM2.2. Eighteen of these metaphases showed signal on one or both chromatids of chromosome 9 in the region 9p13-p21; 95% of this signal was at 9p13.1 (Fig. 1). There was a total of 11 non-specific background dots observed in these 25 metaphases. A similar result was obtained from hybridization of this probe to 15 metaphases from the second normal male and from hybridization using the probe TPM2.4 to 20 metaphases from the 3rd normal male (data not shown).

The localization of TPM2 to 9p13 should be of interest to laboratories positional cloning inherited muscle disorders, especially any that have already linked such diseases to this region.

Acknowledgments

This work was supported by the Neuromuscular Foundation of Western Australia (P.A., S.W., N.L.); the J.H. & J.D. Gunn Medical Research Foundation, the National Health & Medical Research Council of Australia (P.A., N.L., H.E., E.B., D.C.); and the Edith Cowan University Research Fund (C.H., C.M.).

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Figure Legend

Figure 1 Metaphase showing FISH with the probe TPM2.2. a) Normal male chromosomes stained with propidium iodide. Hybridization sites on chromosome 9 are indicated by arrows. b) The same metaphase as (a) stained with DAPI for chromosome identification.