5-1-1996

Mutation Analysis of Fibrillin-2 (FBN2) and Microfibril Associated Protein-3 (MFAP-3): Two Genes Associated with Congenital Contractural Arachnodactyly (CCA), also known as Beal's Syndrome

Darcie Babcock
Portland State University

THESIS APPROVAL

The abstract and thesis of Darcie Babcock for the Master of Science in Biology were presented May 1, 1996, and accepted by the thesis committee and the department.

COMMITTEE APPROVALS: [redacted]

Cheryl Maslen, Co-Chair

[redacted]

Debbie Duffield, Co-Chair

[redacted]

Lester Newman, Co-Chair

[redacted]

Robert Millette

[redacted]

Dirk Iwata-Reuyl
Representative of the Office of Graduate Studies

DEPARTMENT APPROVAL: [redacted]

Leonard Simpson, Chair
Department of Biology

* *

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ABSTRACT

An abstract of the thesis of Darcie Babcock for the Master of Science in Biology presented May 1, 1996.

Title: Mutation analysis of fibrillin-2 (FBN2) and microfibril associated protein-3 (MFAP-3): two genes associated with congenital contractural arachnodactyly (CCA), also known as Beal's syndrome.

Congenital Contractural Arachnodactyly (CCA), also known as Beal's syndrome, is an autosomal dominant disorder characterized by multiple congenital joint contractures, arachnodactyly, dolichostenomelia, and scoliosis with only rare ocular or cardiovascular involvement. CCA has been linked to the fibrillin-2 (FBN2) gene located on chromosome 5q23-31. The phenotype of CCA is similar to Marfan syndrome (MFS) which is caused by defects in the fibrillin-1 (FBN1) gene located on chromosome 15. Fibrillin-1 and fibrillin-2 are components of extracellular matrix (ECM) elastic microfibrils. The linkage studies performed on families affected with CCA suggest that another gene in the area of FBN2 could also be responsible for CCA. Microfibril associated protein-3 (MFAP-3), another microfibril protein gene, has been localized to chromosome 5q32-33.2, the region of FBN2.

This study involves mutation analysis of five patients affected with CCA, three of whom are representative of families affected with CCA. Mutation analysis was performed by chemical mismatch cleavage (CMC) analysis and nonisotopic RNase cleavage assay (NIRCA) analysis on both FBN2 and MFAP-3 cDNA. Prior to this study

only two mutations in FBN2 have been reported in two isolated patients with CCA and none have been reported for MFAP-3. The two mutations reported in FBN2 have not been confirmed in other affected family members. Mutation analysis by CMC completed in this study did not reveal any mutations in either FBN2 or MFAP-3. Reanalysis by NIRCA revealed two mutations in FBN2. One mutation which results in the skipping of exon 31 occurs in an intron and its location is presently unknown. The other mutation, a G to C transversion at nucleotide 3340, predicting a histidine substitution for an asparagine, is a mutation at the -1 position of the 5′ splice site of an intron which results in partial exon skipping although it is unknown whether exon 25 or 26 is skipped. The missense mutation and partial exon skipping result in two different forms of mutant fibrillin-2 molecules. Both of the mutations are present in patients with additional affected family members. Characterization of these mutations will confirm the cosegregation of FBN2 mutations with the CCA phenotype.

MUTATION ANALYSIS OF FIBRILLIN-2 (FBN2) AND MICROFIBRIL ASSOCIATED PROTEIN-3 (MFAP-3): TWO GENES ASSOCIATED WITH CONGENITAL CONTRACTURAL ARACHNODACTYLY (CCA), ALSO KNOWN AS BEAL'S SYNDROME.

by

DARCIE BABCOCK

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1996

ACKNOWLEDGMENTS

I would like most of all to thank my thesis advisor, Dr. Cheryl Maslen, without whom this would truly have never been possible. Her limitless knowledge, support and patience never went unnoticed.

I would also like to thank Dr. Robert Glanville, our esteemed collaborator at Shriner's Hospital for Crippled Children, and the other members of Cheryl's lab, specifically Paul Rupp, for their help and moral support. Don't worry, Paul, this will happen to you, too. I only hope that I will be as much help to you as you have been to me.

Many thanks also to the members of my thesis committee who were essential in making this collaborative effort not only a reality but also a great success.

Finally I would like to thank my family and dear friend Amy for their support and faith in me, and also for fielding those panicked phone calls, assuring me that all of the sacrifices I was making were indeed worth it.

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Introduction

Congenital Contractural arachnodactyly (CCA), also known as Beals syndrome, was first described as a disorder distinct from Marfan syndrome (MFS) in 1971 by Rodney K. Beals and Frederick Hecht. CCA is an autosomal dominant disorder characterized by multiple congenital joint contractures, arachnodactyly (long, thin fingers), dolichostenomelia (long, thin limbs), scoliosis, and abnormality of the external ears, usually referred to as "crumpled" ears (Beals and Hecht, 1971). The congenital joint contractures present in CCA are at their maximum at birth and in some cases lessen as the patient ages (Hecht and Beals, 1972). CCA has a phenotype similar to MFS, with many cases of MFS reported in the early literature actually thought now to be cases of CCA including the first case reported by Marfan in 1896 (Hecht and Beals, 1972). However, CCA lacks ocular problems and aortic root dilitation which are the more serious traits associated with MFS. Because CCA lacks aortic root dilation, it is not a fatal disorder and therefore is not associated with early fatalities as seen in Marfan syndrome.

MFS has been shown to result from a variety of defects in the fibrillin-1 (FBN1) gene located on chromosome 15 (Maslen and Glanville, 1993). Linkage analysis has shown a close association between CCA and the fibrillin-2 (FBN2) gene located on chromosome 5 (Lee et al., 1991; Tsipouras et al., 1992). The cDNA for FBN2 was sequenced in 1994 (Zhang et al., 1994). The coding region of FBN2 is approximately 11 kb (kilo base pairs), slightly longer than that of FBN1. FBN1 and FBN2 have high sequence homology which may explain the phenotypic relationship between MFS and

CCA. Figure 1 is a diagrammatic representation of the amino acid sequence of FBN2, indicating the different domains of the gene. The proteins produced by FBN1 and FBN2 are both present in elastin-associated extracellular matrix (ECM) microfibrils. The fibrillins are high molecular weight, single polypeptide, non-collagenous glycoproteins which are highly insoluble. The tissue distributions of fibrillin-1 and fibrillin-2 are similar, with some distinct differences (Zhang et al., 1994). One notable difference is that fibrillin-2 is present in higher amounts in ECM microfibrils that are rich in elastic fibers such as in the elastic cartilage in the external ear. This would correspond to the ear malformations seen in CCA but not seen in MFS. Other differences in tissue distribution suggest the fibrillins have related but distinct functions (Lee et al., 1991). Linkage analysis and tissue distribution have resulted in FBN2 becoming the focus of studies of CCA. To date there have been only two mutations found in FBN2 in two isolated patients with CCA (Putnam et al., 1995). No shared mutations have been found in families affected with CCA. Consequently, an inheritance pattern demonstrating cosegregation of the CCA phenotype with a FBN2 mutation has yet to be presented.

Very few mutations have been found in either FBN1 or FBN2 using a variety of mutation detection techniques. FBN1 and FBN2 have high G-C to A-T ratios and it has been speculated that mutation analysis of the genes has been difficult due to their G-C rich structures. Single stranded conformational polymorphism (SSCP) analysis has detected only approximately 25% of the mutations in FBN1 found in patients with MFS and less than 20% of the mutations in FBN2 found in patients with CCA. These poor results have made it difficult to prove that FBN2 mutations cause CCA.

The LOD scores for linkage between CCA and FBN2 are 6.2 at both $\theta = 0.000$ and $\theta = 0.001$ (Tsipouras et al., 1992). LOD scores refer to a statistical calculation of the probability that the gene responsible for certain phenotypic characteristics is linked to a polymorphism that is being used as a genetic marker. The calculation is the $\log_{10}$ of the ratio of the probability that the loci are linked, to the probability that they are not linked. These scores suggest the possibility that a gene near FBN2 could also play a key role in CCA. MFAP-3 (microfibril associated protein-3) is another microfibril protein whose gene has been localized to chromosome 5q32-33.2, near the region of FBN2 (Abrams et al., 1995). MFAP-3 has no known homology with any other known proteins. The coding region of MFAP-3 is 1089 bp consisting of two exons with one intron. MFAP-3 is known to localize to elastic microfibrils. Little else is known about the tissue distribution of MFAP-3 to date as it has only been cloned and sequenced recently. Due to the close proximity between FBN2 and MFAP-3 and the lack of mutations implicating FBN2 as the cause of CCA, it is possible that MFAP-3 could be also be associated with CCA.

For this study I proposed mutation analysis of five patients affected with CCA, three of whom be long to families affected with CCA. This study of CCA was unique in that it was the first to include a family affected with CCA. Mutation analysis was performed by chemical mismatch cleavage (CMC) analysis and nonisotopic RNase cleavage assay (NIRCA) analysis on both FBN2 and MFAP-3. While CMC is becoming a widely used mutation analysis system, its use has not been reported by others in the analysis of either FBN2 or MFAP-3. NIRCA has not yet been used for mutation analysis of any genes in a published study.

Chemical mismatch cleavage (CMC) is a heteroduplex mutation analysis method that has been reported to have the ability to detect single base changes in large DNA fragments (Cotton et al., 1988; Grompe et al., 1989; Forrest et al., 1991; Mathew, 1991; Grompe, 1993). CMC has been found to be one of the most sensitive of all of the mutation analysis methods designed so far with the possibility of detecting 100% of point mutations and small deletions or rearrangements. CMC is also the mutation detection system that is the least constrained by size with the possibility of analysis of a1.7 kb fragment at one time. The large size of fragment that can be analyzed by CMC makes it ideal for the detection of small mutations such as point mutations in very large genes.

The principle behind CMC is that a radioactive wild-type DNA and non-radioactive mutant DNA are denatured and annealed together to form a heteroduplex. This heteroduplex is chemically modified with one of two different chemicals at the sites of base pair mismatches and then cleaved with piperidine at the site of the chemical modification. The resulting product is electrophoresed and analyzed with identically treated normal heteroduplexes to reveal the presence of a mutation and its location within the sequence to within 30 bp. Heteroduplexes are modified with osmium tetroxide for mismatched thymines and hydroxylamine for mismatched cytosines. By using separately radiolabeled sense and anti-sense strands, all mismatch combinations can be located. The exact location of the mutation can therefore be found by direct sequencing of only a small area of the gene. DNA-RNA heteroduplexes can also be analyzed. Non-radioactive methods have been developed, but radioactivity is found to be more sensitive in the detection of mutations versus false-positives, such as areas of compressions in the DNA.

Compressions are areas where the DNA has formed secondary structures. These areas are usually G-C rich areas. Besides the use of radioactivity, the chemical modifiers used for CMC are highly toxic which is one of the drawbacks of this method.

NIRCA, or Nonisotopic RNase Cleavage Assay, is a mutation analysis method based on RNase A cleavage assays of RNA:DNA heteroduplexes (Myers et al., 1985) and transcribed genes (Winter et al., 1985) that was developed by Ambion Inc. In these assays, RNase A was found to cleave a single base pair mismatch in a radiolabeled RNA probe that had been hybridized to a DNA target encompassing a point mutation. The resulting fragments were analyzed using a polyacrylamide gel. NIRCA is a modification of this technique that detects mismatches between RNA:RNA hybrids by RNase cleavage using wild-type and mutant RNA transcripts (Figure 2). PCR primers are generated which have the T7 promoter sequence (5′-TAATACGACTCACTATAGGG-3′) attached to the sense or anti-sense primer and the SP6 promoter sequence(5′-ATTTAGGTGACACTATAGGA-3′) attached to the opposing primer. PCR products are generated from cDNA using these primers. The RNA transcripts are then made by *in vitro* transcription of the PCR products using either T7 or SP6 polymerase. Hybrids are produced by combining wild-type and mutant sense and anti-sense RNA strands. The hybrids are cleaved with RNase and the resulting fragments are run on an agarose gel. By comparing the mutant samples with control samples, point mutations can be detected as aberrant bands on the agarose gel. NIRCA, like CMC, can be used to analyze larger fragments of DNA then other mutation analysis techniques with the optimal fragment size for NIRCA being between 500 to 1000 bp. Also like CMC, NIRCA can localize the

mutation within the fragment analyzed, so once a mutation is found only a portion of the fragment needs to be sequenced instead of the entire fragment. Unlike CMC, however, NIRCA cannot predict the exact type of base pair mismatch, but as sequencing is necessary for both methods to locate the exact position of the mutation, this is not a detriment to the system. NIRCA has distinct advantages as a method of mutation analysis because, unlike most of the other mutation detection systems, it doesn't require the use of radioactive materials or toxic chemicals for analysis. NIRCA has also been found to be faster and more sensitive when compared to other mutation detection systems when used to detect known mutations. NIRCA has been tested in G-C and A-U rich targets and has been found to be highly effective in detecting single base pair mutations.

Potential mutations located in either FBN2 or MFAP-3 were characterized by sequencing PCR products generated for the area of the suspected mutation. Mutations found by sequencing were confirmed in the patient and, if possible, in other family members by ASO (allele specific oligonucleotide) analysis (Mathew, 1991; Maslen et al., 1995). Normal controls were also screened to prove that the suspected mutations were indeed mutations and not polymorphisms.

ASO analysis is a system of mutation detection that will confirm a single base pair change. ASO analysis is useful to confirm single base pair changes when these changes do not alter any restriction enzyme sites. ASO probes are radiolabeled oligonucleotides that differ from the normal sequence by only one base pair corresponding to the mutation. A pair of oligonucleotides are designed; one which perfectly matches the normal sequence and one which perfectly matches the mutant sequence. ASO probes must be large enough

to be unique within the target genome so they do not hybridize randomly. However, random hybridization is not a major concern because PCR makes the target sequence very specific. ASO probes must also be small enough to detect a single base change because the hybridization of large probes to their target is not affected by small differences in sequence or small changes in hybridization temperature but hybridization of small probes to their target is easily disrupted by even a single base pair change or a change in the temperature as small as a few degrees. ASO probes are therefore designed to be small, between 11 bp and 20 bp in length, to insure their temperature dependent nature. PCR product encompassing the suspected mutation is generated and bound to a nylon or nitrocellulose membrane. The membrane is hybridizeded with one of the radiolabeled ASO probes at a time. After hybridization, it is washed at a temperature specific to the sequence of the probe. If the probe does not match the target sequence exactly hybridization of the probe will be disrupted because the hybridization will not be thermally stable. A positive signal will result if the mutation that matches the ASO is present on either one or both of the patients alleles. If the suspected mutation is not present, no signal will appear, which is interpreted as a negative signal. Therefore, an ASO that matches the normal sequence can be used as a negative control when hybridized to an allele with a sequence mutation. ASO probes can be used to confirm that a mutation is homozygous or heterozygous, a virtue that was not necessary for this study as all mutations were known to be heterozygous because CCA is an autosomal dominant disorder. This, however, can be a factor in mutation analysis and confirmation in other genes.

Materials and Methods

Patient Samples. Patient samples were provided as blood, tissue, or cultured fibroblast samples through collaboration with Rod Beals, Beat Steinmann, and Uta Franke. RNA and DNA samples from five patients affected with CCA were available for this study. Three of these patients are from families affected with CCA. One family, denoted as Family 1, patients 1-I-1, 1-II-1, and 1-II-2, is a well characterized CCA family that has been followed for a number of years (Beals and Hecht, 1971). Patient 1-I-1 from this family is the proband, the patient originally described with the disorder, and patients 1-II-1 and 1-II-2 are siblings and are patient 1-I-1's offspring. Family 2, denoted as patients 2-I-1 and 2-I-2, are siblings. Family 3 consists of patient 3-I-1, the proband, patients 3-I-2 and 3-I-3, the proband's siblings, and patient 3-II-1, the offspring of patient 3-I-2. Patient 4-I-1 and 5-I-1 are the only patient from their respective families that were available. All of these patients were characterized as being affected by several of the phenotypic characteristics of CCA by persons qualified to make such a judgment before they were received. Only the phenotypic characteristics of the patients from Family 1 were made available. Blood samples were obtained using EDTA as an anticoagulant and were used directly for DNA extraction. Tissue samples were cultured as fibroblast cell lines and grown for RNA and DNA extraction by collaborators at Shriner's Hospital. Control RNA and DNA used were on hand in the laboratory or were isolated from samples of individuals known to have no phenotypic characteristics of CCA.

DNA Isolation. DNA was prepared from blood when possible using the white blood cell nuclei (Bell et al., 1981) or from fibroblast cultures (Ausubel et al., 1995). DNA was prepared from blood by centrifuging the blood at 900 x g for 15 minutes in a Beckman table top centrifuge to separate the plasma from red and white blood cells. The white blood cells were collected and mixed with BCL (blood cell lysis) buffer to lyse the white blood cell membranes. The white blood cell nuclei were pelleted by centrifugation at 900 x g for 20 minutes in a Beckman table top centrifuge and the resuspended in NL (nuclear lysis) buffer to digest the nuclear membranes. Proteinase K/SDS solution was added to the digested white blood cell nuclei and the mixture was incubated at 37° C with shaking for 12-24 hours. Proteinase K/SDS addition digests the proteins present in the cell. The nucleic acids were extracted from the digested proteins using separate phenol and chloroform/isoamyl alcohol (25:1) extractions centrifuging after each extraction at 900 x g for 10 minutes and were precipitated using 0.15M sodium acetate in 95% ethanol. The DNA was pelleted by centrifuging at 2000 x g for 5 minutes in a Beckman table top centrifuge, dried in a Savant Speed Vac, and resuspended in TE at 4° C overnight with shaking. The DNA was reprecipitated by adding 7.5M ammonium acetate and 95% ethanol. The DNA was pelleted by centrifuging at 2000 x g for 5 minutes in a Beckman table top centrifuge and washed with 95% ethanol, repeating the centrifugation. The DNA was dried in a Savant Speed Vac and resuspended in TE-4 at 4° C overnight with shaking.. The concentration of DNA in solution was determined by UV spectrophotometer. The DNA was stored at 4°C.

DNA was isolated from cultured fibroblast cells by trypsinizing the adherent fibroblast cells with 1X Trypsin/EDTA, collecting the cells by centrifugation at 500 x g for 5 minutes in a Beckman table top centrifuge and washing the cells with PBS, repeating the centrifugation. The cells were then mixed with digestion buffer and incubated overnight at 50°C, with shaking to digest the cellular membranes and proteins. The nucleic acids were then extracted using a phenol/chloroform/isoamyl alcohol (25:24:1) extraction centrifuging at 1400 x g for 10 minutes in a Beckman table top centrifuge. RNA was digested by incubation at 37°C for 1 hour with a combination of 30μg of RNase A and 3μg of RNase T1 and the phenol/chloroform/isoamyl alcohol extraction was repeated. The DNA was precipitated using 7.5M ammonium acetate and 100% ethanol. The DNA was pelleted by centrifuging at 1400 x g for 2 minutes in a Beckman table top centrifuge and washed with 70% ethanol, repeating the centrifugation. The DNA was dried and resuspended in TE-4 at 4°C overnight with gentle shaking. The concentration of the DNA in solution was determined by UV spectrophotometer. The DNA was stored at 4°C.

RNA Isolation. RNA was prepared from cultured fibroblasts by cesium chloride gradient separation (Ausubel et al., 1995). Adherent cultured fibroblast cells were trypsinyzed with 1X Trypsin/EDTA, collecting the cells by centrifugation at 500 x g for 5 minutes in a Beckman table top centrifuge and washed with PBS, repeating the centrifugation. The cells were then digested with GIT solution for 5 minutes at room temperature and passed through an 18 gauge needle 10 times to shear the genomic DNA. The mixture was layered over cesium chloride and centrifuged overnight in an ultra-centrifuge at 27,500

rpm. The cesium chloride was drained and the RNA pellet, located at the bottom of the ultra-centrifuge tube, was resuspended in DEPC treated sterile, deionized water and precipitated using 3M sodium acetate and 95% ethanol. The RNA was pelleted by centrifuging at 14,000 rpm for 30 minutes in a micro-centrifuge. Residual cesium chloride was removed by vortexing the pellet with 70% isopropanol/30% 0.2M sodium acetate and spinning at 14,000 rpm for 10 minutes. The RNA was dried in a Savant Speed Vac and resuspended in DEPC treated sterile, deionized water. The concentration of RNA in solution was determined using a UV spectrophotometer. The RNA was stored at -80°C.

cDNA Synthesis. cDNA was synthesized from 20 μg of total RNA in a 20μl reaction volume using random hexamers as a reverse transcription primer (Perkin Elmer Cetus) and Super Script Reverse Transcriptase (BRL). The RNA was reduced to a volume of 10μl by precipitation with 3M sodium acetate and 95% ethanol. The RNA was pelleted by centrifuging at 14,000 rpm for 30 minutes in a micro-centrifuge and washed with 70% ethanol, spinning at 14,000 rpm for 10 minutes. The RNA was dried in a Savant Speed Vac and resuspended in DEPC treated sterile, deionized water, mixed with 50 ηmol of random hexamers, and heated to 70°C for 10 minutes. The mixture was cooled quickly on ice, 4μl of 5X reaction buffer (BRL), 2μl 0.1M DTT, and 1μl 10mM mixed dNTP stock (Perkin Elmer Cetus) was added, and the mixture was heated to 37°C for 2 minutes. 40 Units of the RNase inhibitor RNasin (Promega) and 200 Units Super Script Reverse Transcriptase were added and the mixture was incubated at 37°C for 1 hour. 10μg of

RNase A was added to digest any residual RNA and the mixture was incubated at 37°C for 30 minutes. The cDNA was stored at -80°C.

Mutation Analysis

Chemical Mismatch Cleavage. Mutation analysis was performed by chemical mismatch cleavage, with some modifications as follows (Cotton et al., 1988; Grompe et al., 1989; Mathew, 1991). First and second round PCR was performed using primers listed in Table 1 on normal and mutant cDNA synthesized from total RNA. Oligonucleotide primers were designed using Oligo 5.0 Primer Analysis Software (National Bioscience, Inc.). PCR was performed in a 100μl reaction volume using 2.5U *Taq* DNA polymerase (BRL), 10μl of 10X PCR buffer (BRL), 4μl 50mM $MgCl_2$, 16μl 10mM mixed dNTP stock, and 100μM final concentration of PCR primers unless otherwise indicated. The thermal profile for first round PCR was a two step PCR profile: 94°C for 1 minutes, 68°C for 5 minutes for 35 cycles with a 72°C extension for 5 minutes. The thermal profile for the second round PCR was 94°C for 1 minutes, X°C for 2 minutes, 72°C for 3 minutes for 30 cycles with a 72°C extension for 5 minutes. The specific annealing temperatures for the second round PCR primers are listed in Table 1. Second round PCR products were gel purified by electrophoresing using 5X gel loading buffer on a 0.7% agarose gel containing 0.5μg/ml ethidium bromide at 40 Volts, excising the fragment, and isolating the fragment with Millipore Ultra-free MC filter units using the manufacturer's protocol. PCR

fragments were quantitated using the ethidium bromide dot method (Sambrook et al., 1989).

Second round PCR primers were labeled by heating 10ρmol of primer with 2.5μl 10X labeling buffer in a 25μl reaction volume at 70°C for 10 minutes followed by quick cooling on ice. 10μl 5X labeling buffer, 100μCi 32P γATP (6000Ci/mmol, NEN), 0.5M DTT, and 10 Units PNK were added in a 50μl reaction volume and heated at 37°C for 30 minutes. An additional 10 Units of PNK was added and the mixture was heated for an additional 30 minutes at 37°C. The reaction was heat inactivated at 65°C for 15 minutes. 10ρmol of the opposite (sense or anti-sense) primer was added to its complimentary labeled primer and the primer pair was cleaned using a Microcon-3 filter (Amicon) according to the manufacturer's protocol. Second round PCR was performed using the labeled primer pair and 1μl of the first round PCR product following the above second round thermal profiles. Radiolabeled PCR products were gel purified and quantitated as above.

Heteroduplexes were formed using 100-150ηg of normal or mutant product and 10ηg of radiolabeled normal product with 10x annealing buffer in a 30μl reaction volume. The reaction was boiled for 5 minutes and then heated at 65°C for 1 hour. The reaction was precipitated by adding 100% ethanol and pelleted by centrifuging at 14,000 rpm for 30 minutes in a micro-centrifuge. The heteroduplexes were dried and resuspended in 13μl sterile, deionized water. One half of the heteroduplex was treated with hydroxylamine and the other half with osmium tetroxide before piperidine cleavage of both

reactions. Hydroxylamine cleavage was performed by adding 20μl of hydroxylamine solution, pH 6.0 to 6μl of the heteroduplex and incubating at 37°C for 30 minutes. Osmium tetroxide cleavage was performed by adding 10μl of 2.5X osmium tetroxide buffer, 8μl 4% osmium tetroxide to 6μl of the heteroduplex solution and incubating at 37°C for 20 minutes with mixing every 2-3 minutes. The reactions were stopped by adding 100μl of hot stop buffer and 250μl of 100% ethanol and pelleted by centrifuging at 14,000 rpm for 30 minutes in a micro-centrifuge. The hydroxylamine cleavages were washed twice with 70% ethanol, centrifuging at 14,000 rpm for 2 minutes after each wash and the osmium tetroxide cleavages were washed once with 70% ethanol, centrifuging at 14,000 rpm for 2 minutes. The products of both reactions were dried in a Savant Speed Vac and cleaved with piperidine by adding 50μl of 1M piperidene, vortexing briefly, and incubating at 90°C for 30 minutes followed by cooling to 0°C. The piperidine cleavages were carried out in a Biometra TRIO-thermoblock thermal cycler. The cleavages were terminated by adding 3M sodium acetate, pH 5.2 containing 1.6mg/ml tRNA and 100% ethanol and pelleted by centrifuging at 14,000 rpm for 30 minutes in a micro-centrifuge. The cleavage products are washed once with 70% ethanol, centrifuging at 14,000 rpm for 2 minutes. The cleavage products were dried in a Savant Speed Vac and resuspended in 10μl of formamide loading dye (Stop Solution, USB). The cleavage products were vortexed for 10 seconds to resuspend and heated to 95°C for 3 minutes before they were electrophoresed on a 1X TBE /5% polyacrylamide gel at 75 Watts. 2.5μl of the cleavage products were loaded onto the gel and run for 1 hour. At this point another 2.5μl of the

cleavage products were loaded onto the gel and run until the bromophenol blue dye front reached the bottom of the gel. The two different running times were used to give better resolution of both ends of the cleaved fragment.

Radiolabeled molecular weight markers pBR 322/*Msp I* (NEB)and ΦX 174/*Hae III* (NEB) were loaded onto the gel with the samples. Markers were radiolabeled by end-labeling by adding 2.5μl of 5X exchange buffer (BRL), 50μCi of γ 32P ATP (6000Ci/mmol, NEN), and 10Units polynucleotide kinase (BRL) to 250ηg of either marker in a reaction volume of 25μl and incubating at 37°C for 30 minutes and 75°C for 1 minute to inactivate the PNK. Dilutions of the radiolabeled markers were made and loaded onto the gel according to the specific activity required. The gel was then dried and exposed to autoradiography film at -80°C.

NIRCA. Mutation analysis was also performed by NIRCA (Ambion Inc.) following the manufacturer's protocol. First round PCR products from CMC were used and second round PCR was performed using primers listed on Table 2. Oligonucleotide primers were designed using Oligo 5.0 Primer Analysis Software (NBI). PCR was performed in 25μl reaction volume using 0.5μl of first round PCR product as a template with 2.5U *Taq* DNA polymerase (BRL), 10μl of 10X PCR buffer (BRL), 4μl 50mM $MgCl_2$, 16μl 10mM mixed dNTP stock, and 100μM final concentration of PCR primers unless otherwise indicated. The thermal profile for the second round PCR was 94°C for 25 seconds, X°C for 20 seconds, 72°C for 40 seconds for 30 cycles with a 72°C extension for 5 minutes.

The specific annealing temperatures for the second round PCR primers are listed in Table 2. Gels were run using a 5μg per lane of a 100 bp marker (Promega) that was diluted in the NIRCA RNase digestion buffer and gel loading buffer.

Sequencing. Areas suspected to have a mutation after chemical mismatch cleavage analysis were PCR amplified from a larger PCR fragment using either a forward or reverse CMC second round primers that were biotinylated. See Table 1 for primer and cycle profile. The primers were biotinylated using the Oligonucleotide Biotin Labeling kit (NEB). 750 ρmol of primer was biotin labeled following the manufacturer's protocol. The biotinylated primers were purified ProbeQuant Columns (Pharmacia) following the manufacturer's protocol. The concentration of the biotinylated primers were determined using a UV spectraphotometer. PCR was performed using one biotinylated primer and the complimentary non-biotinylated primer. These reactions were identical to the above non-biotinylated PCR except that the biotinylated primer had a final concentration of 50μM and the non-biotinylated primer had a final concentration of 100μM. The biotinylated strands were separated from the non-biotinylated strands for sequencing using M-280 streptavidan magnetic beads (Dynal, Inc.) following the manufacturer's protocol except that 90μl of PCR product instead of 40μl PCR product as the Dynal protocol suggests. All solution amounts in the manufacturer's protocol were scaled up for the use of 90μl of PCR product. The biotinylated strand, attached to the M-280 streptavidin beads, was resuspended in 7μl of sterile, deionized water prior to sequencing. The non-biotinylated strand was precipitated following the manufacturer's protocol, resuspended in 7μl of

sterile, deionized water. The biotinylated strand was sequenced while remaining attached to the streptavidin beads. Biotinylated and non-biotinylated strands were sequenced by the dideoxy chain termination sequencing method (Sanger et al., 1977) using the Sequenase Version 2.0 kit (NEB) or the Fidelity Sequencing kit (Oncor) following the manufacturer's protocol. Sequencing primers were designed using Oligo 5.0 Primer Analysis Software (NBI). See Table 3 for sequencing primers. The manufacturer's protocol for Fidelity (Oncor) was not modified. The manufacturer's protocol for Sequenase (NEB) was modified as follows: 4 ρmol of primer was used per sequencing reaction, the extension reaction was carried out for 2 minutes at 20°C, and the termination reaction was carried out for 10 minutes at 50°C. The biotinylated strand sequencing reactions were then separated from excess primer and reaction components using a Dynal magnet, resuspended in 6 μl of sterile, deionized water and 4 μl Stop Solution (USB), and heated to 95°C to remove the reaction from the magnetic beads prior to loading the reactions onto a polyacrylamide gel. The non-biotinylated strand sequencing reactions were heated to 75°C for 2 minutes prior to loading onto the polyacrylamide gel. 2.5 μl of each reaction were loaded onto a 5% Long Ranger (J.T. Baker)/0.6X TBE polyacrylamide gel and run at 85 Watts for 1-3 hours as needed for the particular mutation. The sequence was dried and exposed to autoradiography film at room temperature.

ASO Analysis. Single base pair mutations that were located by sequencing were confirmed by ASO analysis. PCR was performed on 250ηg of genomic DNA using primers listed on Table 5. PCR primers were designed using Oligo 5.0 Primer Analysis

Software (NIB). The PCR was performed in 50µl reactions with 2.5U *Taq* DNA polymerase (BRL), 15.5µl of gelatin PCR buffer (Rigat et al., 1992), 10µl 10mM mixed dNTP stock, and 100µM final concentration of PCR primers unless otherwise indicated. The thermal profile for the second round PCR was 94°C for 1 minute, X°C for 2 minutes, 72°C for 3 minutes for 40 cycles with a 72°C extension for 5 minutes. The specific annealing temperatures for the PCR primers are listed in Table 4. 10µl of PCR product was combined with 40µl of sterile, deionized water, 2µl 10N NaOH, and 1µl 0.5M EDTA and heated to 100° C for 10 minutes. The mixture was blotted onto a pre-wetted, positively charged nylon membrane (Boehringer Mannheim) using a Dot Blotting apparatus (BioRad) following the manufacturer's protocol. The blot was baked at 80°C in a vacuum oven for 2 hours to fix the DNA to the nylon. To check the amounts of cDNA bound to the membrane, the blots were stained with a 0.02% methylene blue solution for 10 minutes and de-stained with deionized water three times for 15 minutes each. The blots were then hybridized with a end-labeled ASO. See Table 5 for ASO probes used. The radiolabeled ASO probe was added to the blot in 5 mls of Zeta probe hybridization solution and hybridized overnight at 42°C. The blot was rinsed once with 6X SSC/0.1% SDS at room temperature, washed twice with 6X SSC/0.1% SDS at room temperature, and washed twice with 6X SSC/0.1% SDS at X°C. See Table 6 for wash temperatures for specific ASO probes). The blot was wrapped in saran wrap and exposed to autoradiography film at -80°C.

Results

Mutation Analysis

CMC Analysis. FBN2 was analyzed as seven overlapping fragments of approximately 1.5 kb for CMC analysis due to the size constraint of 1.7 kb of the CMC system. MFAP-3 was analyzed by CMC as one piece due to its smaller size fitting the size constraints of the CMC system. Only one member of each family was analyzed in cases where multiple family members were available. Both the sense and anti-sense strands of the patient's cDNA were analyzed to insure that no mutations were missed. CMC gels were scored as a positive result on the basis of autoradiography bands appearing in the patient lanes which did not appear in the control lane. Positives could appear as a band in either a hydroxylamine or an osmium tetroxide lane. A band in a hydroxylamine lane indicated a cytosine mismatch and a band in an osmium tetroxide lane indicated a thymine mismatch. Positives appeared at a distance from either the 5′ or 3′ end of the fragment depending on the cDNA strand. For example, a positive signal on the gel in the sense strand lane would indicate that the mutation was located some certain distance from the 5′ end of the fragment. The positives could be assigned a localized area in the gene based on their distance from the end of their specific fragment.

Four positives were found for the FBN2 gene. All positives were found in the hydroxylamine lanes. Positives for FNB2 were found at 350 bp from the 5′ end of the first fragment for patient 4-I-1, 650 bp from the 5′ end of the second fragment for patient 1-I-1, 315 bp from the 3′ end of the second fragment for patient 3-I-1, and 350 bp from the 5′

end of fragment six for patients 1-I-1, 2-I-1, 3-I-1, and 4-I-1. These positives were localized to the areas encompassing nucleotides 485 for patient 4-I-1, 2010 for patient 1-I-1, 2545 for patient 3-I-1, and 7180 for patients 1-I-1, 2-I-1, 3-I-1, and 4-I-1 (Figure 3).

NIRCA Analysis. FBN2 and MFAP-3 were analyzed as overlapping fragments of 700-800 bp for NIRCA analysis. NIRCA analysis was scored as a positive if a band or multiple bands were present on the agarose gel in the patient sample lanes that were not present in the control lane. Both the sense and anti-sense strands of the patient's transcribed RNA were analyzed to ensure that no mutations were missed. NIRCA analysis will give a localization of the mutation from either the 5′ or 3′ end of the fragment. NIRCA analysis will not however indicate whether the mutation is a particular distance from the 5′ or 3′ end of the fragment. Further NIRCA analysis is necessary to localize the exact mutation location relative to the 5′ or 3′ end of the fragment. NIRCA analysis was performed on a region of FBN2 that contained a known polymorphism. The normal control was known to be homozygous for the polymorphism, patient 1-II-1 was a known heterozygote, and patient 1-I-1 was known to be homozygous for the wild-type. Patient 1-I-1 was used as the heteroduplex control. The duplex of 1-I-1:1-I-1 showed no bands, the duplex 1-I-1:normal control showed two intense bands at 350 bp and 500 bp, and the duplex 1-I-1:1-II-1 showed two less intense bands at 350 bp and 500 bp (Figure 4). These bands would indicate a mutation that was causing RNase cleavage 350 bp from either the 5′ or 3′ end of the fragment. Results from the NIRCA analysis of the remainder of FBN2 and MFAP-3 are pending.

Sequencing. Sequencing was carried out as described in the Materials and Methods section using primers listed on Table 3. Initial sequencing was carried out using Sequenase v2.0 (USB) and repeat sequencing was carried out with the Fidelity Sequencing system (Oncor).

FBN2, nucleotide 485. Sequencing of the area encompassing the mutation at nucleotide 485 in patient 4-I-1 revealed two transitions at nucleotides 486 and 487 (Figure 5). Both transitions were located in exon 4 of FBN2 and were cytosine to thymine substitutions. The first transition at nucleotide 486 would result in a silent polymorphism with no amino acid substitution. The second transition at nucleotide 487 would result in the substitution of a tyrosine for the expected histidine. Repeat sequencing after ASO analysis revealed these transitions to be a partial compression band and not point mutations.

FBN2, nucleotide 2010. Sequencing of the area encompassing the mutation at nucleotide 2010 in patient 1-I-1 revealed two transversions at nucleotides 2026 and 2030. Both transversions were located in exon 15 of FBN2 and were guanine to cytosine substitutions. The first transversion at nucleotide 2026 would result in a glutamine substituted for the expected glutamic acid. The second transversion at nucleotide 2030 would result in an alanine substituted for the expected glycine. The first transversion would be more detrimental that the second as the first is a change from an acidic amino acid to a neutral amino acid while the second is an exchange of two neutral amino acids.

Repeat sequencing revealed these transversions to be sequence artifact and not point mutations.

FBN2, nucleotide 2545. The sequencing of the area encompassing the mutation at nucleotide 2545 revealed no point mutations. It did however reveal a compression band in the patient sample which was not present in the control sample. Compression bands can be detected as mismatches by CMC. This mutation possibility was discarded from further analysis.

FBN2, nucleotide 7180. The sequencing of the area encompassing the mutation at nucleotide 7180 revealed a transition at nucleotide 7197 (Figure 6). The transition at this position was located in exon 57 of FBN2 and resulted in a thymine to cytosine substitution which results in a silent polymorphism with no amino acid substitution. Further sequencing of patients 1-II-1 and 1-II-2 confirmed this polymorphism. Patients 1-I-1, 1-II-2, and 4-I-1 were homozygous for the wild-type sequence. Patients 1-II-1 and 2-I-1 were heterozygous. Patient 3-I-1 and the normal control were homozygous for the polymorphism. Patients 2-I-2 and 5-I-1 were not sequenced for this area.

ASO Analysis. ASO analysis was performed as detailed in the Materials and Methods section using PCR primers listed on Table 4 and oligonucleotide probes listed on Table 5. Assigning positive signals was based on the presence of a signal dot on the radiography film based on the amount of the sample loaded. Positives could be intense indicating the sequence probed for was present on both alleles or faint indicating the sequence probed for was present on only one allele. Negatives were assigned where there was no signal on

the radiography film in the area of a patient or control sample. Loading of the samples was assessed by Methylene blue staining of the dot blot prior to hybridization with oligonucliotide probes.

FBN2, Exon 4. ASO analysis was carried out on patient 4-I-1 and 50 normal controls. All samples produced a visible signal of approximately equal intensity based on their loading for the normal oligonucleotide probe. None of the samples, including the patient sample, produced a visible signal for either the polymorphic probe, the mutant probe, or the combined polymorphic/mutant probe.

FBN2, Exon 15. ASO analysis was carried out for patients 1-I-1, 1-II-1, 1-II-2 and 50 normal controls. All samples produced a visible signal of approximately equal intensity based on their loading for the normal oligonucleotide probe. None of the samples, including the patient sample, produced a visible signal for either the polymorphic probe, the mutant probe, or the combined polymorphic/mutant probe.

Discussion

Mutation Analysis

CMC Analysis. The positive CMC result for FBN2 in the area of nucleotide 7180 was the first positive found in the CMC analysis of either FBN2 or MFAP3. It was suspected that any change in this area was the result of a polymorphism rather than a disease associated mutation because evidence from the mutation analysis of FBN2 (Putnam et al., 1995) has not shown a universal mutation for all CCA patients. It is more probable that a commonly occurring polymorphism would be present in either one copy or both for the majority of the patients analyzed. The positive signal for patient 1-I-1 was more intense than the signal for the other three patients. After sequencing revealed the change in the sequence to be a polymorphism at nucleotide 7197 occurring in the normal control and five of the patients, it was noted that the intensity of the CMC signal could correspond to the sequence change occurring as a heterozygote or homozygote. When patient 1-I-1, a homozygous wild-type individual, was hybridized with the normal control, a homozygote for the polymorphism, the CMC signal was very intense. On the contrary, when patient 4-I-1, a heterozygote for the polymorphism, was duplexed with the control, a less intense but still positive result was seen. These results would indicate that CMC is a sensitive enough technique to detect a heterozygous mutation. The heteroduplex between patient 2-I-1 and the control would also have been expected to be very intense which is not the case as seen in Figure 3. This sample proved to be extremely difficult to analyze for all of the fragments of FBN2 and, therefore, less of this sample was loaded onto the CMC gels

with respect to the other samples. The reason for the difficulty in analysis of this patient may be related to the poor quality of the fibroblast cells, which did not grow as well as expected. The RNA and DNA isolations were difficult for this sample and the purity of the RNA and DNA with respect to proteins and cellular debris was questionable.

The polymorphism at nucleotide 7197 became a valuable resource, as it was used to optimize the conditions of the CMC technique. A review of the CMC literature published to date shows that the incubation times of the modification of the heteroduplexes with hydroxylamine and osmium tetroxide to be arbitrary. If the chemical modifiers are incubated with the heteroduplex for too long of a time period aberrant nonspecific bands could occur in hyper-sensitive regions of the sequence. These aberrant bands could be mistaken for actual mismatches, wasting valuable time and resources in sequencing areas containing no mutations. Conversely, if the incubation with the modifying chemicals is too short, actual mismatches may be missed because they were not modified. By using heteroduplexes with known mismatches based on the polymorphism at nucleotide 7197, we were able to vary the chemical modification incubation times until we obtained a result that indicated to us the optimal incubation time that would minimize aberrant bands and maximize actual mismatches in the sequence. Once these optimal incubation times were discerned, the rest of the FBN2 gene and the MFAP-3 gene were analyzed by CMC.

It has been noted that compression artifacts can cause false-positives in CMC analysis (Cotton et al., 1988; Grompe et al., 1989; Mathew, 1991). The positive CMC results for FBN2 in the areas of nucleotides 485, 2010, and 2545 were found to be the

results of compression bands causing a false-positive signal. The false-positive result is thought to be the result of modification of the compressed area by virtue of its secondary structure. The chemical modification of the area could be an over-modification and would result in the area being sensitive to cleavage by piperidine. Once a compression is found in the area of a positive CMC signal, it is usually useless to look for any other sequence changes. Therefore, we were not surprised to discover that the area encompassing these three CMC positives contained no mutations.

NIRCA Analysis. NIRCA was performed on the region of FBN2 containing the polymorphism at nucleotide 7197 to test the accuracy of the assay and also to optimize the assay conditions. We needed to know if NIRCA could detect a heterozygote easily and reliably without giving an obscure or difficult to interpret result. The results of NIRCA analysis were positive. NIRCA analysis was able to detect a homozygous mismatch (Figure 4, lanes 1 and 4) at a higher intensity that a heterozygous mismatch (Figure 4, lanes 2 and 5) but the heterozygote detection was still very clear. Three different dilutions of the three RNase stock solutions were used on the polymorphic heteroduplexes: the standard dilution suggested by the manufacturer, a dilution twice that of the standard, and a dilution one-half that of the standard. No discernible differences were noted between the three dilutions. The higher dilution did not result in an increase in aberrant bands and the lower dilution was still able to detect the mismatch although not as intensely as the standard dilution. The remaining fragments of the FBN2 gene and the

MFAP3 gene are being analyzed presently using the standard dilution of the RNase stocks and no data is available for them.

Sequencing. PCR products are difficult to sequence because it is difficult to maintain single strands which are necessary for sequencing. PCR products can be denatured but will readily reanneal making it nearly impossible to anneal the sequencing primer to the strand of interest to continue the sequencing procedure. This difficulty was overcome by biotinylating one of the PCR primers used to generate the DNA fragment to be sequenced. The biotinylated strand of the PCR product can then be separated from the non-biotinylated strand by magnetic separation using streptavidin coated magnetic beads utilizing the interaction between streptavidin and biotin. Using this procedure the PCR product was separated into single strands that could be sequenced by dideoxy-chain termination sequencing.

Compression bands, partial compression bands, and sequence artifacts which are aberrant bands appearing on the sequencing gel are common problems with sequencing DNA. PCR products add another level of difficulty by introducing amplified sequence that may not be complete at the 5′ and 3′ ends or may have errors in the sequence that have been introduced by the PCR procedure itself. The difficulties of sequencing PCR products that may have incomplete 5′ and 3′ ends can be overcome by the use of sequencing primers that lie within the PCR fragment and do not overlap with the PCR primers or by using "nested" PCR primers that lie within the area of the first PCR primers. The "nested" PCR primers are used in a second round of PCR. The difficulties that arise

from PCR artifact are not as easily solved. These artifacts can be present in either the normal control or the patient sample. Repeating the PCR and the sequencing reactions will usually yield the correct sequence because it is unlikely that a random PCR error would be introduced on two separate occasions. Compression bands cause areas of the DNA sequence that are not separated into single base pairs during sequencing. Compressions result in a solid line that is present in all four lanes of the sequencing gel at one time. They can be present for a single base pair or for several bases in a series. *Partial compressions are compression bands seen in two or three lanes at one time but not* all of the lanes as a normal compression. Compressions and partial compressions must be dealt with by either changing the sequencing conditions or the sequencing system that is being used. Different sequencing systems make use of different enzymes to carry out the sequencing procedure and these enzymes will react with different aberrations of the DNA in different ways. Some enzymes and temperature conditions will sequence through a difficult area with little or no trouble where others may result in partial or full compressions.

One indication of sequence artifact or partial compression bands is the presence of more that one transition or transversion occurring in succession or relatively near another. Initial sequencing with the Sequenase v2.0 kit (USB) for FBN2 in the regions of nucleotides 485 and 2010 both had two sequence alterations within a few base pairs of each other. The sequences would indicate that the regions contained a mutation and a polymorphism in the same area which is not impossible but is unlikely. When ASO analysis revealed that there were no changes to the sequence in these areas, the fragments

were sequenced with the Fidelity Sequencing kit (Oncor). The Fidelity kit was used because it uses T4 DNA polymerase instead of T7 DNA polymerase used by Sequenase v2.0 and higher temperatures. The Fidelity kit was able to sequence through the partial compressions to reveal normal sequence. These areas were discounted as areas of mutation in FBN2. The secondary sequencing of these areas proves that the ASO analysis was working properly to detect single base changes in the sequence. ASO analysis is still considered to be a reliable method of mutation confirmation. Initial sequencing with the Sequenase v2.0 kit (USB) for FBN2 in the region of nucleotide 2545 revealed no point mutations and a compression band. The compression was in the correct area to allow for a CMC positive signal so the area was also discounted as an area of mutation in FBN2.

Conclusions. To date, this study has not revealed any mutations in the FBN2 or MFAP-3 genes that are associated with CCA. It did, however, reveal one polymorphism that was used to optimize the NIRCA system. As no mutations have been found in families affected with CCA, an inheritance pattern demonstrating cosegregation of the CCA phenotype with a FBN2 or MFAP-3 mutations cannot be established. FBN2 and MFAP-3 cannot be discounted as the gene or genes associated with CCA. CMC has not been shown to be an effective mutation analysis system for either of these genes. This could be a result of the high G-C to A-T content of FBN2. The G-C to A-T content of MFAP-3 has not been calculated and could be a factor in mutation analysis. FBN2 has been difficult to analyze by SSCP and has now been seen to be resistant to CMC analysis.

NIRCA analysis appears to be useful for mutation analysis of FNB2 and is currently being used for that purpose (Note added in proof: see Appendix I).

Sequencing of PCR products is a difficult task which can been made appreciably easier by the use of biotin labeled primers and streptavidin beads. When used with internal sequencing primers and a reliable sequencing method, the sequencing of PCR products becomes a relatively easy task. This biotin-PCR sequencing technique has become an important technique in this study and other studies we have conducted in other areas. ASO analysis is a proven technique for mutation confirmation and holds great promise for use with FNB2 when mutations are located as a rapid mutation detection technique.

APPENDIX I

ADDENUM

Abstract submitted to the 4th International Symposium on the Marfan Syndrome, Davos, Switzerland, August 11-14, 1996. The abstract refers to patients 2-I-1 and 2-I-2.

Aberrant splicing of fibrillin-2 in a family with Congenital Contractural Arachnodactyly

Cheryl L. Maslen*, Darcie Babcock*, Michael Raghunath[#],
Beat Steinmann[†]
*Oregon Health Sciences University, Portland, Oregon, USA; [#]University of Münster, Münster, Germany; [†]University Childrens Hospital, Zürich, Switzerland.

Congenital Contractural Arachnodactyly (CCA) is an autosomal dominant disorder that is phenotypically similar to, but genetically distinct from Marfan syndrome. Genetic linkage analysis implicated the fibrillin-2 gene (FBN2) as the CCA locus. Mutation analysis of single CCA patients indicate that defects in FBN2 may be responsible for that disorder. However, co-segregation of a mutant allele with the disease phenotype has not been established. We have investigated the primary cause of CCA in a large, well characterized kindred with four documented generations of affected individuals. Previous studies showed linkage of the CCA phenotype to FBN2. Mutation analysis of the proband's cDNA using Non-Isotopic Rnase Cleavage Assay (NIRCA, Ambion Corp., Austin Texas, USA) identified the presence of a skipped exon that was subsequently identified as exon 31. DNA sequence analysis of genomic DNA identified the splice site alteration responsible for the exon-skipping event. The occurrence of exon skipping was confirmed in the cDNA of an affected sibling. Genomic DNA from 29 additional available family members, both affected and unaffected, was then analyzed for the splice site mutation. The results clearly demonstrate co-segregation of the abberant splice site with the CCA

phenotype. This unequivocally establishes, for the first time, that mutations in FBN2 are responsible for the CCA phenotype.

Abstract submitted to the 4th International Symposium on the Marfan Syndrome, Davos, Switzerland, August 11-14, 1996. The abstract refers to patients 3-I-1, 3-I-2, 3-I-3, and 3-II-1.

A Single Mutation That Results in an Asp→His Substitution and Partial Exon Skipping in a Patient with Congenital Contractural Arachnodactyly

Cheryl Maslen*, Darcie Babcock*, Cheryll Gasner# and Uta Francke#
*Oregon Health Sciences University, Portland, Oregon, USA; #Stanford University Medical Center, Stanford, California, USA.

Recent investigations of the molecular basis of congenital contractural arachnodactyly (CCA) indicate that mutations in the fibrillin-2 gene (FBN2) cause CCA. In order to determine the range and nature of FBN2 mutations associated with CCA, we are examining cRNA from a series of patients using Non-Isotopic Rnase Cleavage Assay (NIRCA; Ambion Inc., Austin, Texas, USA.). A NIRCA positive result indicated the presence of a mutation in cRNA prepared from cultured fibroblasts derived from CCA patient, FB904. The patient has the classic features of CCA; multiple contractures, arachnodactyly, and crumpled ears, with no apparent heart or eye manifestations. This mutation had previously gone undetected when cDNA from this individual was analyzed by chemical mismatch cleavage analysis, indicating that NIRCA may be a more robust technique for detecting fibrillin mutations. DNA sequence analysis of the NIRCA positive region detected a G to C transversion at nucleotide 3340 (G3340C), which predicts the substitution of histidine for asparagine at amino acid residue 1114. This asparagine residue acts as a calcium-binding ligand in the 12th calcium-binding pEGF-like domain. In addition, the G3340C mutation alters the last nucleotide of exon 25; position -1 of the 5'

donor splice site, which is a highly conserved G (77%). Subsequent RT-PCR and DNA sequence analyses demonstrate that this missense mutation also acts as a splice site error, resulting in partial skipping of that exon. Consequently, the complex manifestation of this genotype may result in two different populations of mutant fibrillin-2 molecules as components of elastic microfibrils in one individual. However, the phenotype of the affected individual indicates that if both mutant fibrillin-2 forms are present in the matrix, they are not appreciably less well tolerated than is a single defective molecule.

APPENDIX II

FIGURES

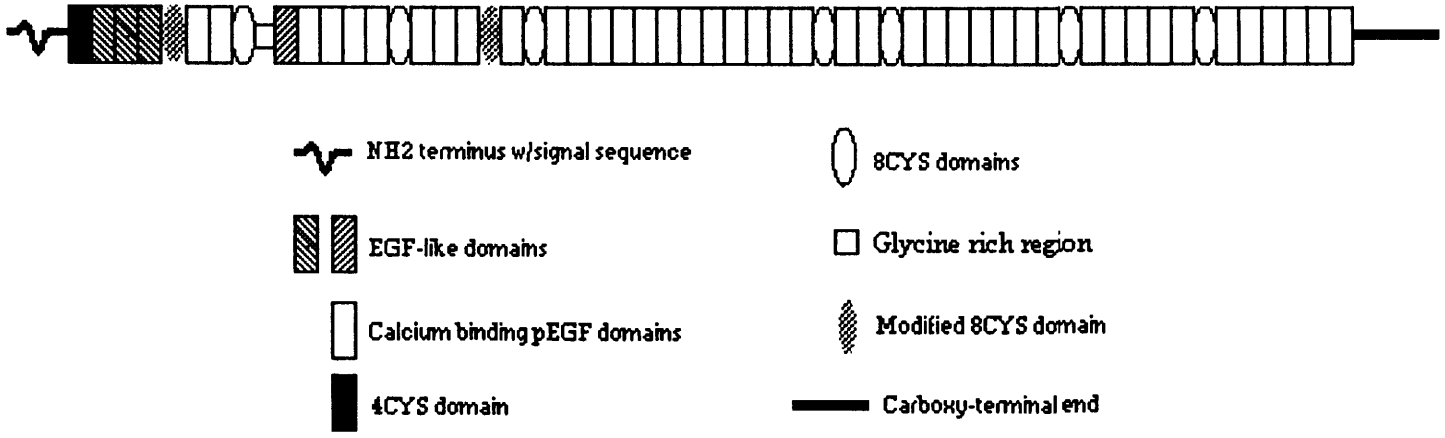

Figure 1. Diagramatic representation of the amino acid sequence of fibrillin-2 indicating the different domains present. Diagram courtesy of Cheryl Maslen.

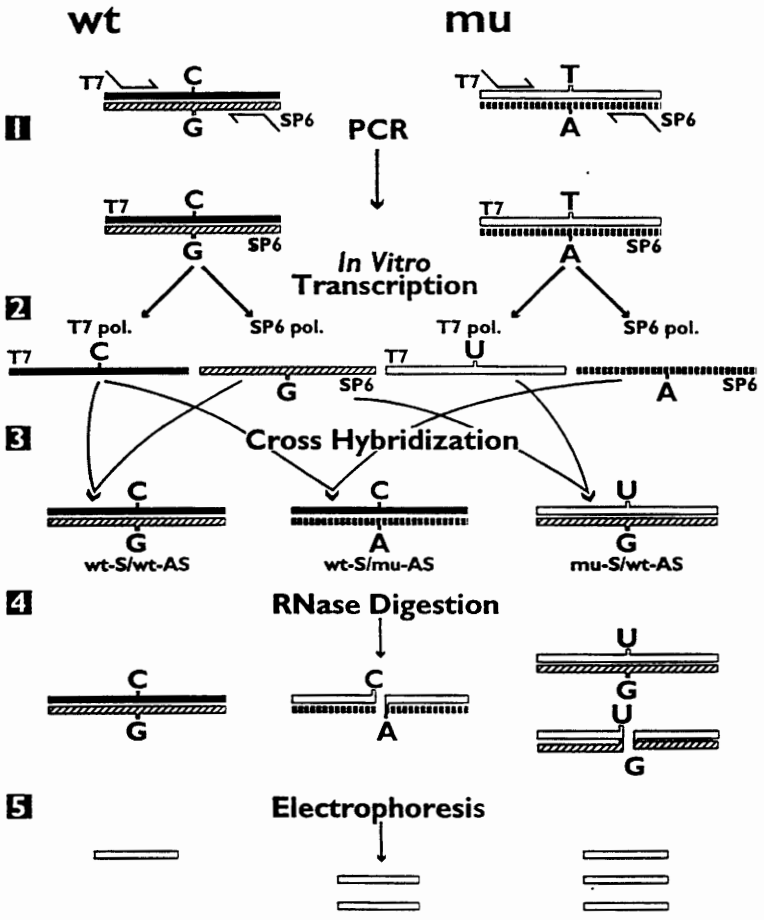

Figure 2. Schematic diagram of the NIRCA methodology from Ambion, Inc.

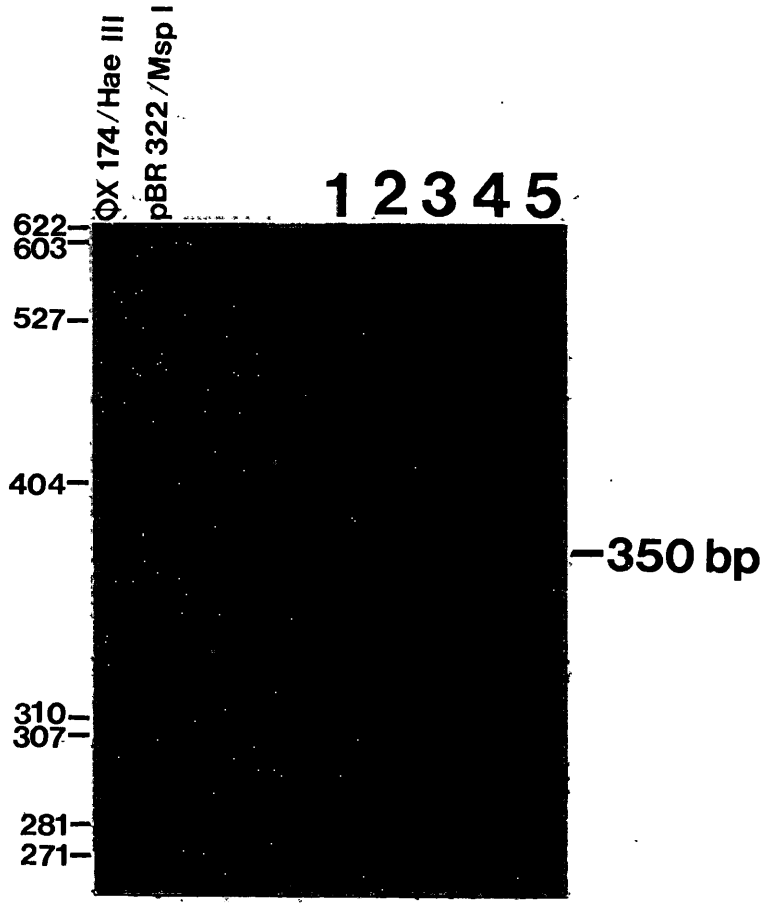

Figure 3. Chemical mismatch cleavage of the area encompassing FBN2 nucleotide 7180. Lane 1 is Control, Lane 2 is Patient 4-I-1, Lane 3 is Patient 1-I-1, Lane 4 is Patient 3-I-1, and Lane 5 is Patient 2-I-1 for hydroxylamine cleavage. The band at ~350 bp indicates a cytosine mismatch for all patients represented. Size markers located to the left of sample lanes are ΦX 174/Hae III and pBR 322/Msp I.

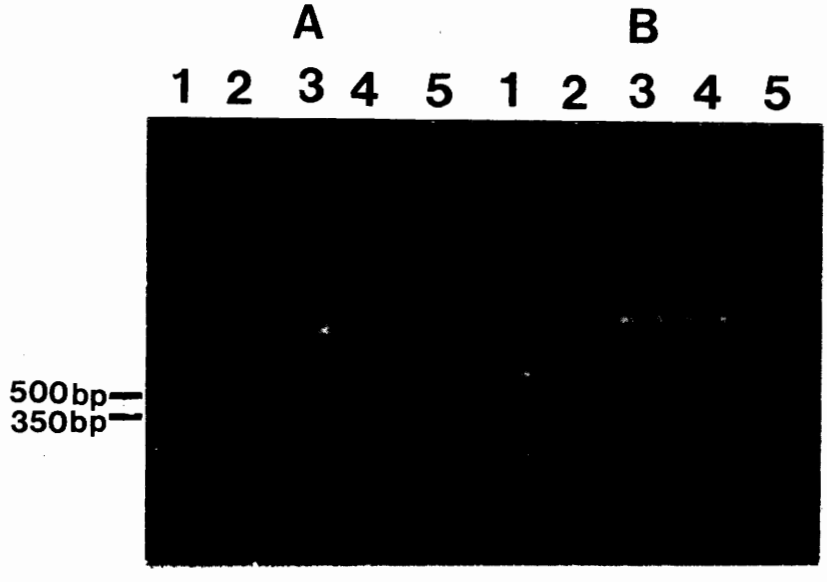

Figure 4. NIRCA analysis of the area of a known FBN2 polymorphism. Side A is an RNase digestion with one-half of the standard dilution of RNase. Side B is an RNase digestion with twice the standard dilution of RNase. Lanes 1 and 4 are Patient 1-I-1:Control, Lanes 2 and 5 are Patient 1-I-1:1-II-1, and Lane 3 is 1-I-1:1-I-1 and serves as a positive control. Lanes 1 and 2 are Patient 1-I-1 SP6 transcribed and Lanes 4 and 5 are Patient 1-I-1 T7 transcribed. Bands present are at 500 bp and 350 bp. No markers are present.

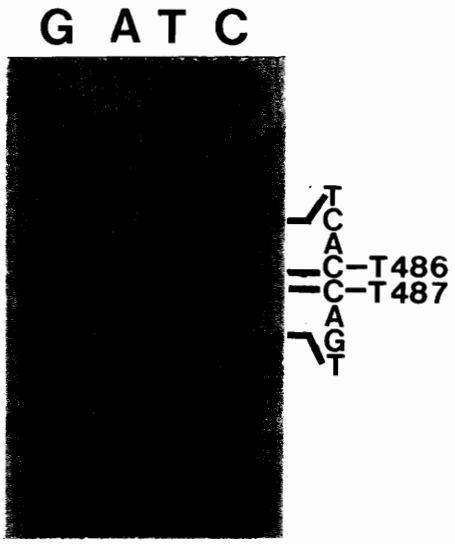

Figure 5. Sequencing of the area encompassing FBN2 nucleotide 485 for patient 4-I-1. Nucleotides 486 and 487 indicate cytosine to thymine substitutions. Sequencing was performed with the Sequenase v2.0 kit (USB).

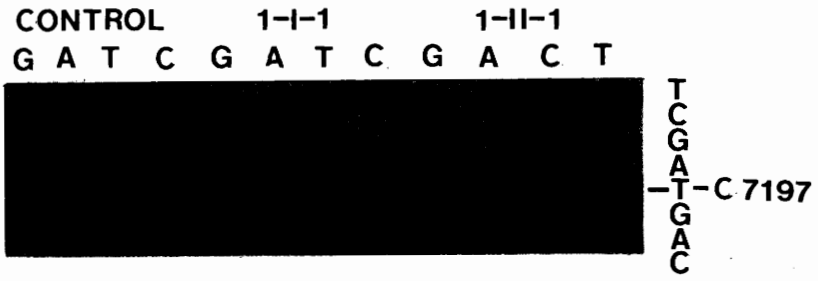

Figure 6. Sequencing of the area encompassing FBN2 nucleotide 7180 for Control, patient 1-I-1, and patient 1-II-1. Nucleotide 7197 indicates thymine to cytosine substitution, resulting in a silent polymorphism. Sequencing was performed with the Sequenase v2.0 kit (USB).

APPENDIX III

GENERAL ABBREVIATIONS AND SOLUTIONS

Abbreviations

DEPC:	Diethylpyrocarbonate.
DTT:	Dithiothreitol.
EDTA:	Ethylenediaminetetraacetic acid.
GIT:	Guanidinium isothiocyanate solution.
PBS:	Phosphate buffered saline.
PNK:	Polynucleotide kinase.
rpm:	Revolutions per minute.
SDS:	Sodium dodecyl sulphate.
SSC:	Standard saline citrate.
Tris:	Tris(hydroxymethyl)aminomethane.
UV:	Ultra violet.

Solutions

DNA and RNA Isolations

BCL (blood cell lysis) buffer:	0.32M sucrose, 10mM Tris-Cl, pH 7.5, 5mM $MgCl_2$, 1% Triton X-100
NL (nuclear lysis) buffer:	75mM NaCl, 24mM EDTA, pH 8.0
Proteinase K/SDS:	NL buffer, 0.2mg/ml proteinase K, 0.9% SDS
TE:	10mM Tris-Cl, pH 8.0, 1mM EDTA, pH 8.0
TE-4:	10mM Tris-Cl, pH 8.0, 0.1mM EDTA, pH 8.0
1X PBS (per liter):	8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4
Digestion buffer:	100mM NaCl, 10mm Tris-Cl, pH 8.0, 25mM EDTA, pH 8.0, 0.5% SDS, 0.1mg/ml proteinase K
GIT:	4M guanidinium isothiocyanate, 20mM sodium citrate, 0.4% n-lauryl-sarcosine
Cesium chloride:	5.7M cesium chloride, 100mM EDTA, pH 8.0

cDNA synthesis

Mixed dNTP stock (10mM):	10mM dGTP, 10mM dATP, 10mM dTTP, 10mM dCTP

Mutation Analysis

50X TAE:	2M Tris, 50mM EDTA, ph 8.0, 5.7% glacial acetic acid
10X Labeling buffer:	200mM Tris-Cl, 10mM spermidine, 1mM EDTA, pH 8.0
5X Labeling buffer:	250mM Tris-Cl, 50mM $MgCl_2$, 25mM DTT, 25% glycerol
10X Annealing buffer:	3M NaCl, 30mM Tris, 35mM $MgCl_2$

Hydroxylamine solution, pH 6.0:	1.39g hydroxylamine, 1.6mls sterile deionized water, 1ml diethylamine
2.5X Osmium tetroxide buffer:	25mM Tris, pH 7.7, 4mM EDTA, pH 8.0, 3.75% pyridine
Hot stop buffer:	0.2M NaCl, 10mM Tris-Cl, pH 7.7, 1mM EDTA, pH 8.0, 5μg/μl tRNA
10X TBE:	1.1M Tris, 1.1M boric acid, 20mM EDTA, pH 8.0
Gel loading buffer:	1mM EDTA, pH 8.0, 25mg Bromophenol Blue, 50% glycerol

ASO Analysis

Zeta Probe(BioRad):	0.22M Na_2HPO_4, pH 7.2, 0.5M NaCl, 14% SDS, 2mM EDTA, pH 8.0, 50% formamide
20X SSC (pH 7.0):	3M NaCl, 0.3M sodium citrate
Gelatin PCR Buffer:	100mM $MgCl_2$, 30mM Tris-Cl, pH 8.4, 167mM KCl, 0.33μg/ml gelatin

APPENDIX IV

TABLES

TABLE 1 FRAGMENT	CMC PRIMERS SENSE PRIMER (5'-3')	ANTI-SENSE PRIMER (5'-3')	ANNEALING TEMPERATURE
FBN2 First Round			
1	ACAGGTTCGGTCCGCTACAGC	GCTTACAGATATCTATTGTCTGGTTC	68°C
2	AGTGGCAATGGCAATGGCTATGG	GCCAGGGAACACCTCACACTCAT	68°C
3	CTGTTGGCTCAACATCCAGGAC	CGTCCAGATCAATACACTTGATGC	68°C
4	GATGTACAGATGTGGATGAGTGTG	TGATGCAGTCTGCATTCCGCTG	68°C
5	GTGTGCATTAACCAGATTGGCAGT	GATGCACATGAAGGTGCCGATTAG	68°C
6	TCCTATGAATGCACGTGCCCGAT	CGTTGATTTTGCACTCGTAGCATG	68°C
7	GCAATTACGGCTGCTCTAACACG	TCAGCTGCCTACAGTACCATGAG	68°C
MFAP-3 First Round	GGTTCTCTACTCACATCT	CTTCTAAGAAACAGGTTCC	49.9°C
FBN2 Second Round			
1	GTTCGGTCCGCTACAGC	GTCCAGTGATGATAGGTC	53.8°C
2	ACAGGCTTCATCCCCAT	TCTTCACACGTAACACCT	52.8°C
3	TCAACATCCAGGACAGC	TGATGCCGTTTCCAATC	54.9°C
4	TGGTGCTCATAACTGCG	TATCACCATTGCTGCAC	53.8°C
5	GCTGTGAATGCCCTACA	CTAGATTCACAGTCGTG	50.8°C
6	CTATGAATGCACGTGCCCGA	TCTGGGGACAGAGCAT	54.8°C
7	GCTACCTCTGTGGCTG	TACCATGAGGACGCAG	51.7°C
MFAP-3 Second Round	ATGAAGCTACATTGTTGC	TTACAGCTGACAGTTTTC	51.1°C

Table 2 **NIRCA PRIMERS**

FRAGMENT	SENSE PRIMER (5'-3')	ANTI-SENSE PRIMER (5'-3')	ANNEALING TEMPERATURE
FBN2			
1/1	GATAATACGACTCACTATAGGG CTCTGAAGGCGGGTTT	TCATTTAGGTGACACTATAGGA TAGCCTGGCATTCATC	56.2°C
1/2	GATAATACGACTCACTATAGGG CCATCCCTGTGAGAT	TCATTTAGGTGACACTATAGGA TATCTATTGTCTGGTTC	52.7°C
2/1	GATAATACGACTCACTATAGGG AGGGACAGGCTTCATC	TCATTTAGGTGACACTATAGGA CGCACACACACTCCTT	54.4°C
2/2	GATAATACGACTCACTATAGGG CTGTGGGCATGGATGG	TCATTTAGGTGACACTATAGGA GGGTGGCACAGCATTC	56.0°C
3/1	GATAATACGACTCACTATAGGG AGCCACTCTGAAATCT	TCATTTAGGTGACACTATAGGA AAGCTGCCCTCAGTGT	55.1°C
3/2	GATAATACGACTCACTATAGGG ACATTGACGGATGTGA	TCATTTAGGTGACACTATAGGA TGCCGTTTCCAATCCA	54.9°C
4/1	GATAATACGACTCACTATAGGG TGCTCATAACTGCGAC	TCATTTAGGTGACACTATAGGA TTGTGATGGGGTTAGG	54.7°C
4/2	GATAATACGACTCACTATAGGG AAACCCCTGTGAGACA	TCATTTAGGTGACACTATAGGA GATTATCACCATTGCT	51.9°C
5/1	GATAATACGACTCACTATAGGG CAGTTTCCGCTGTGAA	TCATTTAGGTGACACTATAGGA GTATCAAAGCATCTCC	51.6°C
5/2	GATAATACGACTCACTATAGGG TTCCTGTACTAATACT	TCATTTAGGTGACACTATAGGA TTAGATTCTTACACAT	49.1°C
6/1	GATAATACGACTCACTATAGGG AAGATCAAAAGATGT	TCATTTAGGTGACACTATAGGA CACAGAGGAACTGGCA	52.7°C
6/2	GATAATACGACTCACTATAGGG AGTTATCAGTGTTCA	TCATTTAGGTGACACTATAGGA CAGAGCATTTTCCTCA	53.9°C

7/1	GATAATACGACTCACTATAGGG TATTACAGAGTGGGA	TCATTTAGGTGACACTATAGGA GGGTCAACATTCAAAG	53.4°C
MFAP-3			
1	GATAATACGACTCACTATAGGG AAAATATCTCTACCAA	TCATTTAGGTGACACTATAGGA GTTTTGGCTGAGGTAA	50.0°C
2	GATAATACGACTCACTATAGGG CCATCAATGAGTTCT	TCATTTAGGTGACACTATAGGA GCGAGCTGATTTTGT	52.4°C

TABLE 3 **SEQUENCING PRIMERS**

GENE	PRIMER (5'-3')	DIRECTION	POSITION (bp)
FBN2			
1	GATGGAAGACGCTCCCTGGAG	Sense	299
2/A-F	TGTGTTTGAATGGAATGTGC	Sense	1874
2/A-R	CATTCGGACTTGGTCACTGC	Anti-sense	2180
2/B-F	GTGCCGAAACACGCCAGGAA	Sense	2472
2/B-R	GTCCCCTTCAGGCTGTCAA	Anti-sense	2687
6	CCAGGAATCTGTGAAATGGACTG	Sense	7033
MFAP-3/F	ATGAAGCTACATTGTTGC	Sense	1
MFAP-3/R	TTACAGCTGACAGTTTTC	Anti-sense	1089

TABLE 4 PRIMERS FOR ASO ANALYSIS

EXON	SENSE PRIMER (5'-3')	ANTI-SENSE PRIMER (5'-3')	ANNEALING TEMPERATURE
4	TCAGCAGTGCAGTGTGAG	TCCACAATATGTTCCAAT	49.2°C
15	GATGTTGATGAATGCCAG	ATCCATGCCCACAGCCAG	53.6°C

TABLE 5 ASO PROBES

EXON	ASO SEQUENCE	BASE CHANGE	AMINO ACID CHANGE	WASH TEMPERATURE
4	CAGATGACCACTGCCA	No Change	No Change	50°C
4	CAGATGATCACTGCCA	486 bp	No Change	48°C
4	CAGATCATTACTGCCA	486/487 bp	No Change/Histidine-Tyrosine	46°C
4	CAGATGACTACTGCCA	487 bp	Histidine-Tyrosine	48°C
15	ACAGTGAAGGGTCCT	No Change	No Change	46°C
15	ACAGTCAAGCGTCCT	2026/2030 bp	Glutamic Acid-Glutamine/ Glycine-Alanine	46°C
15	CAACAGTCAAGGGTC	2026 bp	Glutamic Acid-Glutamine	46°C
15	AGTGAAGCGTCCTTC	2030 bp	Glycine-Alanine	46°C

REFERENCES

Abrams, W. R., Ma, R., Kucich, U., Bashir, M. M., Decker, S., Tsipouras, P., McPherson, J. D., Wasmuth, J. J., and Rosenbloom, J. (1995). Molecular cloning of the microfibrillar protein MFAP3 and assignment of the gene to human chromosome 5q32-q33.2. Genomics 26, 47-54.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. (1995). Short Protocols in Molecular Biology, Third ed. (Boston: John Wiley and Sons, Inc).

Beals, R. K., and Hecht, F. (1971). Congenital contractural arachnodactyly. J. Bone Jt. Surg. 53-A, 987-993.

Bell, G. I., Karam, J. H., and Rutter, W. J. (1981). Polymorphic DNA region adjacent to the 5' end of the human insulin gene. Proc. Natl. Acad. Sci. USA 78, 5759-5763.

Cotton, R. G. H., Rodrigues, N. R., and Campbell, R. D. (1988). Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc. Natl. Acad. Sci. USA 85, 4397-4401.

Forrest, S. M., Dahl, H. H., Howells, D. W., Dianzani, I., and Cotton, R. G. H. (1991). Mutation detection in phenylketonuria by using chemical cleavage of mismatch: importance of using probes from both normal and patient samples. Am. J. Hum. Genet. 49, 175-183.

Grompe, M. (1993). The rapid detection of unknown mutations in nucleic acids. Nature Genet. 5, 110-117.

Grompe, M., Muzny, D. M., and Caskey, C. T. (1989). Scanning detection of mutations in human ornithine transcarbamoylase by chemical mismatch cleavage. Proc. Natl. Acad. Sci. USA 86, 5888-5892.

Hecht, F., and Beals, R. K. (1972). "New" syndrome of congenital contractural arachnodactyly originally described by Marfan in 1896. Pediatrics 49, 574-579.

Lee, B., Godfrey, M., Vitale, E., Hori, H., Mattei, M.-G., Sarfarazi, M., Tsipouras, P., Ramirez, F., and Hollister, D. W. (1991). Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. 352, 330-334.

Maslen, C. L., and Glanville, R. W. (1993). The molecular basis of Marfan syndrome. DNA Cell Biol. 12, 561-572.

Maslen, C. L., Babcock, D., and Illingworth, D. R. (1995). Occurrence of a Mutation Association with Wolman Disease in a Family with Cholesteryl Ester Storage Disease. J. Inher. Metab. Dis. 18, 620-623.

Mathew, C. G., ed. (1991). Protocols in Human Molecular Genetics, vol. 9. (Clifton,NJ: Humana Press).

Myers, R. M., Larin, Z., and Maniatis, T. (1985). Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 230, 1242-1246.

Putnam, E. A., Zhang, H., Ramirez, F., and Milewicz, D. M. (1995). Fibrillin-2 (FBN2) mutations result in the Marfan-like disorder, congentital contractural arachnodactyly. Nature Genet. 11, 456-458.

Rigat, B., Hubert, C., Corvol, P., and Soubrier, F. (1992). PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidylcarboxypeptidase 1). Nuc. Acids Res. 20, 1433.

Sambrook, J., Fritsch, E. F., and Maniatis, T., eds. (1989). Molecular Cloning, Second ed. (Cold Springs Harbor, New York: Cold Springs Harbor Laboratory Press).

Sanger, F., Niklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Tsipouras, P., Del Mastro, R., Sarfarazi, M., Lee, B., Vitale, E., Child, A. H., Godfrey, M., Devereux, R. B., Hewett, D., Steinmann, B., Viljoen, D., Sykes, B. C., Kilpatrick, M., and Ramirez, F. (1992). Genetic linkage of the Marfan syndrome, ectopia lentis, and congenital contractural arachnodactyly to the fibrillin genes on chromosomes 15 and 5. New Engl. J. Med. 326, 905-909.

Winter, E., Yamamoto, F., Almoguera, C., and Perucho, M. (1985). A method to detect and characterize point mutations in transcribed genes. Proc. Natl. Acad. Sci. USA 82, 7575-7579.

Zhang, H., Apfelroth, S. D., Hu, W., Davis, E. C., Sanguineti, C., Bonadio, J., Mecham, R. P., and Ramirez, F. (1994). Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. J. Cell Biol. 124, 855-863.