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Elastin: genomic structure and point mutations in patients with supravalvular aortic stenosis

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We describe the complete exon–intron structure of the human elastin (*ELN*) gene located at chromosome 7q11.23. There are 34 exons occupying ~47 kb of genomic DNA. All exons are in-frame, allowing exon skipping without disrupting the reading frame. Microsatellites are located in introns 17 and 18. Deletions of all or large parts of the *ELN* gene have been previously reported in two patients with supravalvular aortic stenosis (SVAS), and SVAS is also a frequent feature of Williams syndrome, where patients are hemizygous for *ELN*. We list primer pairs for amplifying each exon, with flanking intron, from genomic DNA to allow detection of point mutations in the *ELN* gene. We show that some patients with isolated SVAS have point mutations that are predicted to lead to premature chain termination. Knowledge of the genomic structure will allow more extensive mutation screening in genomic DNA of patients with SVAS and other conditions.

INTRODUCTION

The protein elastin is responsible for the characteristic elastic properties of many tissues including skin, lung and large blood vessels. The elasticity of these tissues depends on elastic fibres in the extracellular matrix. These are composed of an amorphous and a microfibrillar component, and the amorphous component (~90% of the mature elastic fibre) is composed of elastin. The human elastin cDNA has been isolated and sequenced (1). Early reports that *ELN* mapped to chromosome 2 proved mistaken; it maps to 7q11.2 (2) and has 34 exons. Comparative studies showed that the human cDNA lacks sequences corresponding to exons 34 and 35 of the bovine elastin gene, so that human exon 34 is homologous to bovine exon 36 (1).

The initial product of the *ELN* gene is tropoelastin, a 72 kDa polypeptide with a characteristic primary structure of alternating hydrophobic and cross-linking domains. The hydrophobic domains are thought to form a floppy structure of stacked β-sheets with β-turns that is responsible for the resilience of the protein. After secretion, individual tropoelastin chains are covalently cross-linked to form a highly insoluble network of elastic fibres. Cross-linking requires oxidation of lysyl residues by the copper-dependent enzyme lysyl oxidase (3–5). Alanine(A)–lysine(K) rich motifs ($A_{3–10}KA_{2–3}K$) occur in the cross-linking domains in exons 15, 17, 19, 21, 23, 27, 29 and 31, and in modified form in exons 6 and 25.

Since elastin is a major component of the aorta and large arteries, disruption of the elastin gene might be expected to cause vascular problems. One candidate condition is supravalvular aortic stenosis (SVAS) (6). SVAS is a congenital narrowing of the ascending aorta. The condition is frequently progressive and may lead to heart failure and early death, so that patients often need corrective surgery in early infancy. SVAS may occur sporadically or as a familial condition with autosomal dominant inheritance (7). Linkage analysis mapped familial SVAS to chromosome 7q (8) and a family in which a translocation t(6:7)(p21.1;q11.23) co-segregated with SVAS showed disruption of the elastin gene at 7q11.23 (9,10). Subsequently large intragenic deletions involving the elastin gene have been reported in two unrelated SVAS patients (11,12). SVAS also occurs as part of Williams syndrome (WS). WS (MIM 194050) is a contiguous gene syndrome caused by a microdeletion at 7q11.23 (13). WS patients are hemizygous for the elastin gene. Affected individuals are mentally retarded but with an unusual cognitive profile (they speak fluently but have poor visuospatial skills). They have dysmorphic facies, frequently heart abnormalities (mainly SVAS), short stature, hyperacusis, and often infantile hypercalcaemia. Other connective tissue phenotypes associated with WS include hernias, hoarse voice, joint abnormalities, and premature ageing of skin.

SVAS is very variable, both in familial dominant SVAS and amongst patients with Williams syndrome. Nickerson *et al.* (14) report that the elastin deletion is seen in ~90% of Williams syndrome patients, and in our experience, all patients with a classic WS phenotype are hemizygous at the elastin locus (unpublished data). Nevertheless, only 5% have severe clinical SVAS. It is unclear to what extent the facial and other features of WS can be attributed to haploinsufficiency for elastin, rather than to deletion or silencing of other genes flanking the elastin locus. It is also unknown what proportion of SVAS patients have

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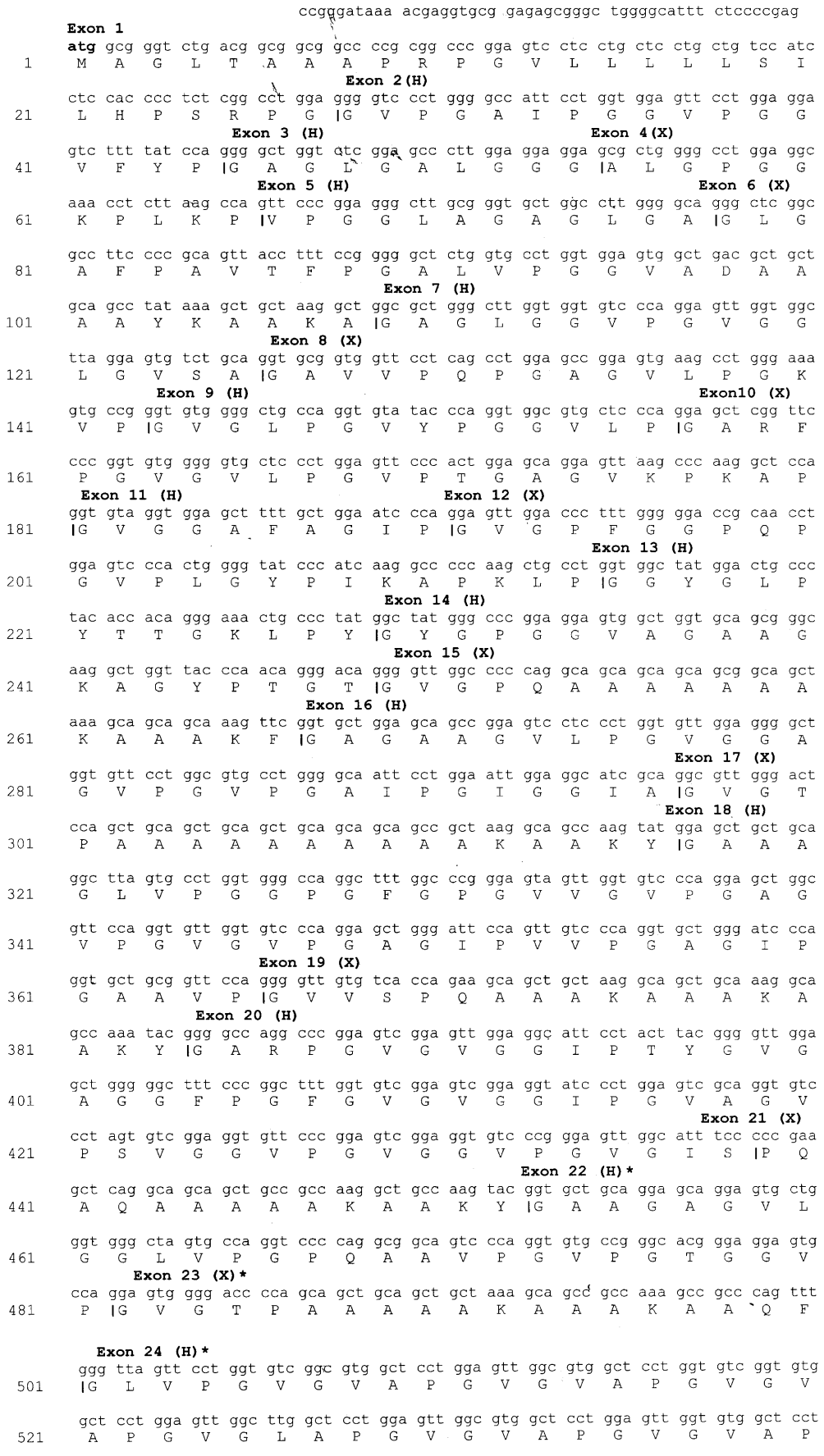
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Exon 1
atg gcg ggt ctg acg gcg gcg gcc ccg cgg ccc gga gtc ctc ctg ctc ctg ctg tcc atc
M A G L T A A A P R P G V L L L L L S I
Exon 2 (H)
ctc cac ccc tct cgg cct gga ggg gtc cct ggg gcc att cct ggt gga gtt cct gga gga
L H P S R P G |G V P G A I P G G V P G G
Exon 3 (H)
Exon 4 (X)
gtc ttt tat cca ggg gct ggt gtc gga gcc ctt gga gga gga gcg ctg ggg cct gga ggc
V F Y P |G A G L G A L G G G |A L G P G G
Exon 5 (H)
Exon 6 (X)
aaa cct ctt aag cca gtt ccc gga ggg ctt gcg ggt gct ggc ctt ggg gca ggg ctc ggc
K P L K P |V P G G L A G A G L G A |G L G
gcc ttc ccc gca gtt acc ttt ccg ggg gct ctg gtg cct ggt gga gtg gct gac gct gct
A F P A V T F P G A L V P G G V A D A A
Exon 7 (H)
gca gcc tat aaa gct gct aag gct ggc gct ggg ctt ggt ggt gtc cca gga gtt ggt ggc
A A Y K A A K A |G A G L G G V P G V G G
Exon 8 (X)
tta gga gtg tct gca ggt gcg gtg gtt cct cag cct gga gcc gga gtg aag cct ggg aaa
L G V S A |G A V V P Q P G A G V L P G K
Exon 9 (H)
Exon10 (X)
gtg ccg ggt gtg ggg ctg cca ggt gta tac cca ggt ggc gtg ctc cca gga gct cgg ttc
V P |G V G L P G V Y P G G V L P |G A R F
ccc ggt gtg ggg gtg ctc cct gga gtt ccc act gga gca gga gtt aag ccc aag gct cca
P G V G V L P G V P T G A G V K P K A P
Exon 11 (H)
Exon 12 (X)
ggt gta ggt gga gct ttt gct gga atc cca gga gtt gga ccc ttt ggg gga ccg caa cct
|G V G G A F A G I P |G V G P F G G P Q P
Exon 13 (H)
gga gtc cca ctg ggg tat ccc atc aag gcc ccc aag ctg cct ggt ggc tat gga ctg ccc
G V P L G Y P I K A P K L P |G G Y G L P
Exon 14 (H)
tac acc aca ggg aaa ctg ccc tat ggc tat ggg ccc gga gga gtg gct ggt gca gcg ggc
Y T T G K L P Y |G Y G P G G V A G A A G
Exon 15 (X)
aag gct ggt tac cca aca ggg aca ggg gtt ggc ccc cag gca gca gca gca gcg gca gct
K A G Y P T G T |G V G P Q A A A A A A A
Exon 16 (H)
aaa gca gca gca aag ttc ggt gct gga gca gcc gga gtc ctc cct ggt gtt gga ggg gct
K A A A K F |G A G A A G V L P G V G G A
Exon 17 (X)
ggt gtt cct ggc gtg cct ggg gca att cct gga att gga ggc atc gca ggc gtt ggg act
G V P G V P G A I P G I G G I A |G V G T
Exon 18 (H)
cca gct gca gct gca gct gca gca gca gcc gct aag gca gcc aag tat gga gct gct gca
P A A A A A A A A A A K A A K Y |G A A A
ggc tta gtg cct ggt ggg cca ggc ttt ggc ccg gga gta gtt ggt gtc cca gga gct ggc
G L V P G G P G F G P G V V G V P G A G
gtt cca ggt gtt ggt gtc cca gga gct ggg att cca gtt gtc cca ggt gct ggg atc cca
V P G V G V P G A G I P V V P G A G I P
Exon 19 (X)
ggt gct gcg gtt cca ggg gtt gtg tca cca gaa gca gct gct aag gca gct gca aag gca
G A A V P |G V V S P Q A A A K A A A K A
Exon 20 (H)
gcc aaa tac ggg gcc agg ccc gga gtc gga gtt gga ggc att cct act tac ggg gtt gga
A K Y |G A R P G V G V G G I P T Y G V G
gct ggg ggc ttt ccc ggc ttt ggt gtc gga gtc gga ggt atc cct gga gtc gca ggt gtc
A G G F P G F G V G V G G I P G V A G V
Exon 21 (X)
cct agt gtc gga ggt gtt ccc gga gtc gga ggt gtc ccg gga gtt ggc att tcc ccc gaa
P S V G G V P G V G G V P G V G I S |P Q
Exon 22 (H)*
gct cag gca gca gct gcc gcc aag gct gcc aag tac ggt gct gca gga gca gga gtg ctg
A Q A A A A A K A A K Y |G A A G A G V L
ggt ggg cta gtg cca ggt ccc cag gcg gca gtc cca ggt gtg ccg ggc acg gga gga gtg
G G L V P G P Q A A V P G V P G T G G V
Exon 23 (X)*
cca gga gtg ggg acc cca gca gct gca gct gct aaa gca gcc gcc aaa gcc gcc cag ttt
P |G V G T P A A A A A K A A A K A A Q F
Exon 24 (H)*
ggg tta gtt cct ggt gtc ggc gtg gct cct gga gtt ggc gtg gct cct ggt gtc ggt gtg
|G L V P G V G V A P G V G V A P G V G V
gct cct gga gtt ggc ttg gct cct gga gtt ggc gtg gct cct gga gtt ggt gtg gct cct
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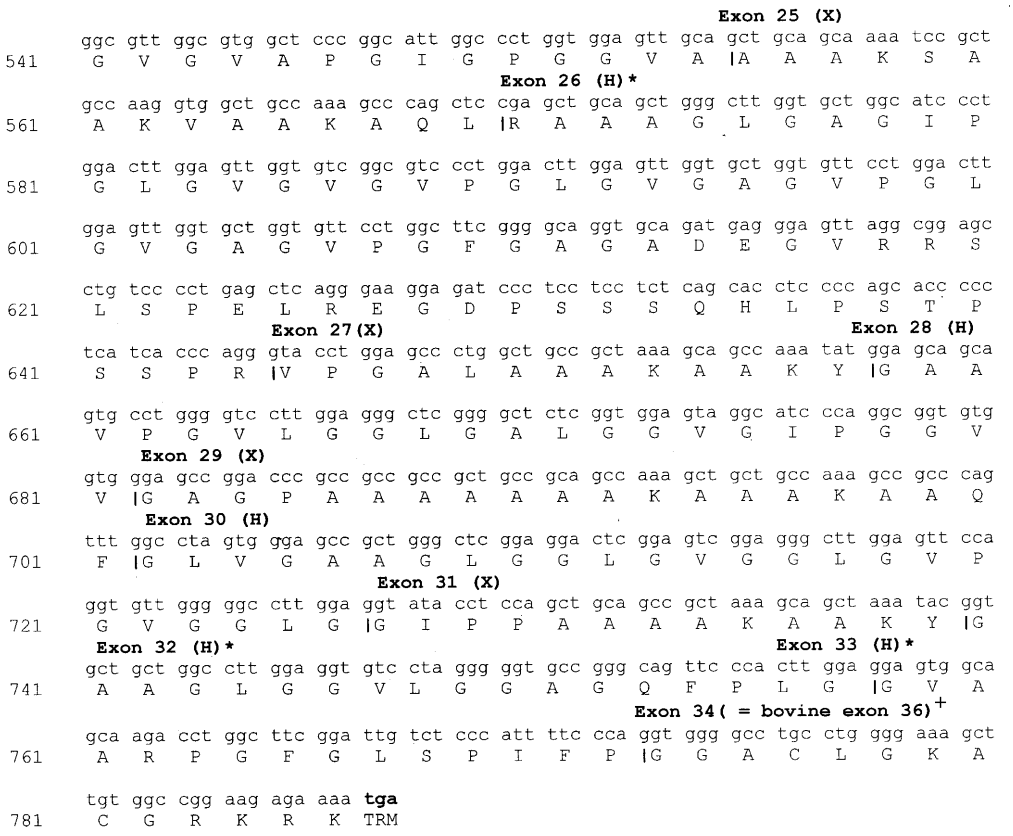

Figure 1. ELASTIN cDNA sequence showing exon–intron boundaries. (H) hydrophobic domain; (X) cross-linking domain; *subject to alternative splicing; + (C-terminus): conserved cysteines and four terminal basic residues.

abnormalities at the *ELN* locus, what types of *ELN* mutations can cause SVAS, and whether other categories of *ELN* mutation might cause different connective tissue problems. To address these issues and allow screening of genomic DNA for mutations, we have determined the complete genomic structure of the human *ELN* gene. Until now, only partial exon–intron structures have been available in the literature (1) or in databases. In this study we have defined the unpublished exon–intron boundaries of the elastin gene and have carried out mutation screening in patients with dominant SVAS. We show that point mutations in the elastin gene can cause SVAS.

RESULTS

Structure of the elastin gene

We isolated a 125 kb bacterial artificial chromosome (BAC) containing the complete *ELN* coding sequence from a commercial library (Genosys), by screening with primers designed from the 5′ and 3′ ends of the published elastin cDNA sequence (1). We used fluorescent *in situ* hybridisation (FISH) to confirm that the BAC mapped to 7q11.23 (data not shown). To define the unpublished exon–intron boundaries of the *ELN* gene, PCR primers were designed randomly using the last published exon boundary from the 3′ end of the human cDNA sequence (1) as a starting point, and PCR products from genomic DNA were sized and sequenced. In cases where no amplification was obtained between a set of primers, the vectorette method was used to define exon boundaries, as described by Riley *et al.* (15). Primers designed from the intronic sequence were used to amplify each exon from genomic DNA, and in each case gave a product of the expected size. Our method of analysis leaves open the possibility that some transcripts might contain additional exons not present in the published cDNA sequence we relied on, such as the bovine exon 34 and 35 sequences that are missing from the published human sequence.

Figure 1 shows the complete *ELN* cDNA sequence with exon–intron boundaries marked, and Table 1 summarises the splice junctions and surrounding sequence in the elastin gene. The coding sequence we have determined agrees with that published by Indik *et al.* (1). It comprises 2361 bp (including the termination codon) split into 34 small exons ranging in size from 30 to 225 bp and extending over ~47 kb of genomic sequence. The introns range from 82 bp to ~10 kb, and all the exon–intron boundaries conform to the published consensus sequences (16). Twenty-seven out of the 33 codons split by introns encode glycine residues (Table 1).

Alu and microsatellite repeats

As previously reported (17), the 3′ region of the gene is rich in Alu sequences. A BLAST search of our sequence showed that introns

Table 1. Structure of the elastin gene

Exon	5' end	Size bp	3' end	Note	Intron size	Intron codon
1		82	CCT GGA G gtaaggacccctcgc		~ 10 kb	G
2	cctgtttccttcag GG GTC CCT	51	TAT CCA G gtaacgtacatgaaa		~ 1.3 kb	G
3	tctctccccccacag GG GCT GGT	30	GGA GGA G gtgagctcagaaacc		~ 1.2 kb	A
4	ctcttccctctgcag CG CTG GGG	33	AAG CCA G gtaagaccaagggct	X	~ 3.5 kb	V
5	gtttcttatccacag TT CCC GGA	36	GGG GCA G gtgagtgctgacacc		~ 2 kb	G
6	tcctcccctccgcag GG CTC GGC	93	AAG GCT G gtgagtggtgctctt	X	276 bp	G
7	gtcctggctctgcag GC GCT GGG	51	TCT GCA G gtacgatggctatcc		82 bp	G
8	ccccttctgtgccag GT GCG GTG	51	GTG CCG G gtcagtgcggaatcc	X	~ 700 bp	G
9	ttcttttggccacag GT GTG GGG	42	CTC CCA G gtgagagcaaggagg		~ 2 kb	G
10	tttgtcccccggcag GA GCT CGG	72	GCT CCA G gtatgcagctgtctg	X	~ 1 kb	G
11	tttttatcattacag GT GTA GGT	30	ATC CCA G gtgaggcaaggctgg		~ 800 bp	G
12	tctttattcccacag GA GTT GGA	72	CTG CCT G gtaagtcagagggac	X	~ 1 kb	G
13	ctgtcctctctccag GT GGC TAT	42	CCC TAT G gtgagtgagaccctt		~ 500 bp	G
14	ctctctgttttgcag GC TAT GGG	60	GGG ACA G gtaaggaaagcctca		~ 400 bp	G
15	gcttccttcccccag GG GTT GGC	54	AAG TTC G gtgagtgcccctgga	X	~ 3 kb	G
16	tccccatctcaacag GT GCT GGA	90	ATC GCA G gtaacatctgtccca		82 bp	G
17	tggctgtgttttcag GC GTT GGG	60	AAG TAT G gtgagtgcctcccgg	X	~ 1.3 kb	G
18	ctttttcttccacag GA GCT GCT	147	GTT CCA G gtgagctgggct(gt)n		~ 1.4 kb	G
19	ttttggtctctccag GG GTT GTG	54	AAA TAC G gtgagtgctatgctg	X	~ 1.6 kb	G
20	cccttccctctgcag GG GCC AGG	165	ATT TCC C gtgagccttagtcac		237 bp	P
21	ctcgtttccttgtag CC GAA GCT	42	AAG TAC G gtaagtgccctgcct	X	~ 700 bp	G
22	tctccactccccgag GT GCT GCA	87	GTG CCA G gtgagctgtgtctcc	*	171 bp	G
23	tcttcacacctccag GA GTG GGG	57	CAG TTT G gtaagtccccctcac	X*	~ 2.7 kb	G
24	ttctcaatcttgcag GG TTA GTT	162	GTT GCA G gtgagtttcatgagt	*	90 bp	A
25	ctctccctccctcag CT GCA GCA	45	CAG CTC C gtgagtgcctcgccc	X	~ 199 bp	R
26	gccctctgtctgcag GA GCT GCA	225	CCC AGG G gtgcatagtaaaatc	*	~ 450 bp	V
27	tttctttctccccag TA CCT GGA	39	AAA TAT G gtgagtgcaccccac	X	~ 2 kb	G
28	ctcctctccccgcag GA GCA GCA	72	GTG GTG G gtgagttgaaacccc		85 bp	G
29	ggtctgtgttcccag GA GCC GGA	60	CAG TTT G gtgagcactgggtgg	X	250 bp	G
30	cagctgtctccacag GC CTA GTG	75	CTT GGA G gtgagagttgttctg		~ 2 kb	G
31	gcctcatctccccag GT ATA CCT	39	AAA TAC G gtgagttcccctctg	X	210 bp	G
32	cctgcctcttctcag GT GCT GCT	54	CTT GGA G gtaggggtggccag	*	~ 750 bp	G
33	aaatctctcctgcag GA GTG GCA	45	TTC CCA G gtatgccaggctccc	*	~ 1.9 kb	G
34	cctcctcccgtccag GT GGG GCC	41	AGA AAA TGA nnnnnnnnn	C		

X = cross-linking domain. *Subject to alternative splicing.
C = C-terminal exon containing conserved cysteines and four basic residues; corresponds to exon 36 of bovine elastin.
The polymorphic microsatellite in intron 18 is indicated as (gt)n. It has 17–20 repeats. A second microsatellite, (AG)n, is present in intron 17 ~230 bp upstream of the 3′ end.

18, 19, 22, 27, 30, 32 and 33 gave high homology with Alu-like sequences, especially Alu J and Alu Sx. Introns 30 (~50% Alu), 32 (~40% Alu) and 33 (~33% Alu) were particularly rich in Alu-like sequences.

Intron sequencing detected two microsatellite repeats. An $(AG)_n$ repeat was located ~230 bp upstream of the 5′ acceptor splice site of exon 18. At 10 bp downstream from the 3′ donor splice site of exon 18 is a $(GT)_{17–20}$ repeat. This latter repeat has been reported previously (2; because of ambiguity in numbering of exons, it was said to be in intron 17), and is polymorphic with heterozygosity 0.626.

Mutations in SVAS patients

Patients with diagnosed SVAS were analysed for mutations in the elastin gene. Intronic primers were designed to amplify each of the 34 exons from genomic DNA (Table 2). Mutations were sought by combined single-strand conformation polymorphism (SSCP) and heteroduplex analysis (18). Where bands of abnormal mobility were seen, the relevant exon was sequenced.

Patient SVAS12 presented at birth with a heart murmur. Cardiac catheterisation was thought to show obstruction, and an initial diagnosis of hypertrophic obstructive cardiomyopathy was made. The patient was seen at another centre at the age of 3 years for assessment of his heart murmur. Echocardiography suggested SVAS on the basis of waisting of the ascending aorta and post-stenotic dilatation. Subsequent cardiac catheterisation revealed a significant pressure gradient (106 mm Hg) between his left ventricle and aorta. At surgery he was found to have severe SVAS, a thickened aortic valve, stenosis at the origin of the right pulmonary artery, subvalvular infundibular pulmonary stenosis and severe left ventricular hypertrophy. He did not have the facial features of WS, and his psychomotor development was normal. Of significance in the family history, his brother died suddenly in the first year of life and at autopsy was noted to have repaired SVAS, repaired central pulmonary artery stenosis and marked ventricular hypertrophy. The aortic valve and proximal aorta were markedly dysplastic with extreme thickening beyond the valve. The proband's mother had presented to cardiologists in childhood with a murmur and a clinical diagnosis of aortic stenosis was made.

SVAS12 showed no evidence of a large deletion: elastin FISH (WSCR probe, Oncor) was normal. Amplified DNA gave an abnormal band in exon 26 on heteroduplex analysis, which was

Table 2. Primer sequences for the amplification of exons 1–34 of the human elastin gene

Exon	Forward PCR primer 5'–3'	Reverse PCR primer 5'–3'	Size of product, bp
1	CAGCCGACGAGGCAACAATTAGGC	TCAGCGTCTAGTCACCTGCAAAGG	228
2	TCCATGTAATTGTGGGTTTTGCC	CAATGTTCCTACCTTCTGTAGTG	256
3	CTTGCCCAAGGTCACGTAGTTAG	ATGAGGGAGTCCTTGATGCTCGG	262
4	GGTTGGATAAGTAGTAGATGGAT	AGGCTAATCGGTGTCCACACCTC	244
5	TCCAGGAGACATTTCCCACTCTG	GGCAGTTGGTATCAGCATCAGTC	264
6	AGGCAGGGCCAGAGCGTAGGAGT	AAGCCTGAGTTGAGGGAAGGTTC	252
7	TACGCAATGCCTCACCTGTCCTG	TGCCCTCTGTCCTCCAGCCCCAG	256
8	TACGCAATGCCTCACCTGTCCTG	TGCCCTCTGTCCTCCAGCCCCAG	256
9	CTGCCTGGGTGGGAAGGGCTG	GGCCTTACTATGATGCCCAGGCT	270
10	GTTCCCAGCAGGGCCTGCAAGGC	GGCCCTGAGCCAGTCCAGGATCC	231
11	CGCAGCATGCGATGACTGGTCTG	GGAGAAATTGGGCAGGCTTGGAT	260
12	GAGATTCAGGGAGTCCCTCGAAG	CACCCGGCCGAGTGGTGCATCTT	239
13	TGGTGGGAGCCCAGCAAGGCATG	AGATTCCCACTCCAAGACCCCAA	229
14	AGTGTGATGTCTGCACAGATGAC	ACCAGGGTCTGGATGCAGGGGTG	225
15	CTGAAGCTCCCATGTATACCCAC	GTTCAATAGTAATGGGAATGGAG	244
16	CGTCTAAGTGGCCATCCTGCCTG	AGCCACAGAGGGTGAGGTCCTTG	239
17	CTGGCGTGCCTGGGGCAATTCCTG	TTGCGGCTAGGGTCTCCGAGGTC	236
18	ATACTCTACTAACCACCCTTCTA	TGGCAATAGTCTCAATATTTCTC	270
19	CACAGATGGGTAGACAGAGGGAT	AGTAGAGGAGGGTCCTTTAGTTC	219
20	CTCTTTCCCAATCCATCAGCATC	CCCATCCCTTCTCAACCCATGTC	295
21	GAGGTCGTATCCATGCCTTACAG	TCCAGGCCATTTCAGTCCTGGAG	253
22	AAAGTGAGTACTGGGAGGGGCAA	CGACCTTGGTCAACTCCAGGGAC	207
23	GTCCCTGGAGTTGACCAAGGTCG	CCCAGAATGTGACAGCTTAAGTG	290
24	AGCTTCTGTCCTCTTTGATCAGG	GGGCCCCTCAGGCTCATTGACTC	252
25	TGGCTCCCGGCATTGGCCCTGGTG	CCTGGCTGTTGCCCCTAACCAGC	238
26	GGCATGCTCCCTGCCTGCTGTCG	CCCAGATGCTTAGGAGAACCTAA	311
27	CAGCTTCAGGGCTTTGAGGAAGC	GTGACCACCCCAGTCCTGTGCTG	260
28	AACACTCATTTTCCCTCCTCTCC	GCCTGGGGGCCTGGCGGCAGCTC	290
29	AACACTCATTTTCCCTCCTCTCC	GCCTGGGGGCCTGGCGGCAGCTC	290
30	CCATCGAAGGCCAGGGGAGACC	CCATCTCTGTCTCGCATACACAC	244
31	GGTGGCATTGGCATTCCTGAGCCG	GTTAAGGAATGTCCCAGACAAGAT	220
32	TTCCTTAACCCAGAACCCAGCAG	CCTTGTGTGGACATGGGCTCTGG	242
33	CCAGACAGAGGTCTTGGGTGAGC	CCCTTCTGAGCAGGAGATGGCAC	233
34	GGGATTAGAGCCGAAACTGAGAG	TTGCCCTGTGGATCTGCAAGCGC	258

not present in 40 non-SVAS controls (Fig. 2). Sequencing revealed insertion of a T base in codon 606 of exon 26, producing a frameshift predicted to cause premature termination 10 codons downstream:

Normal allele:

```
603 GGT GCT GGT GTT CCT GGC TTC GGG GCA GGT GCA GAT GAG
    G   A   G   V   P   G   F   G   A   G   A   D   E
```

Mutant allele:

```
603 GGT GCT GGT TGT TCC TGG CTT CGG GGC AGG TGC AGA TGA
    G   A   G   C   S   W   L   R   G   R   C   R  Stop
```

The mutation was confirmed by designing an allele-specific ARMS primer to allow selective amplification of the mutant allele (19). ARMS amplification and sequencing showed that the proband's mother also has the mutation (Fig. 2).

Patient SVAS50 presented at the age of 8 weeks with a heart murmur and episodes of cyanosis. Echocardiography at 4 months of age showed SVAS and pulmonary arterial stenosis. This was progressive and cardiac catheterisation at 18 months showed a left ventricular pressure of 142 mm Hg with a pull back gradient of 66 mm Hg from left ventricle to aorta at the supravalvular level. There was no gradient at the level of the aortic valve. The right ventricular systolic pressure and branch pulmonary artery pressures were 76 and 15 mm Hg respectively although the catheter wedged very quickly in the branch pulmonary arteries because of the very small size of the branch vessels. Angiography showed the supravalvular aortic narrowing and the very abnormal pulmonary arteries. Corrective open heart surgery was performed at the age of 21 months and at this time it was noted that the aorta and pulmonary arteries were very thick and abnormal. His developmental progress is normal to date and he has no features of Williams syndrome. His mother had cardiac follow up for a heart murmur until the age of 6 years, but a recent echocardiogram showed no evidence of supravalvular aortic stenosis and no pulmonary artery stenosis.

Patient SVAS50 showed no evidence of a large deletion: elastin FISH (WSCR probe, Oncor) was normal. Heteroduplex-SSCP analysis showed a heteroduplex shift in exon 21 which was not present in 43 normal controls. Sequence analysis revealed a single base substitution (CAG→TAG) in codon 442 which changed a glutamine to a stop codon (Q442X). This mutation creates a *Hin*dIII site, allowing us to confirm the mutation by restriction digest analysis:

Normal allele:

```
440 GAA GCT CAG GCA GCA
    E   A   Q   A   A
```

Mutant allele (*Hin*dIII site underlined):

```
440 GAA GCT TAG GCA GCA
    E   A  Stop
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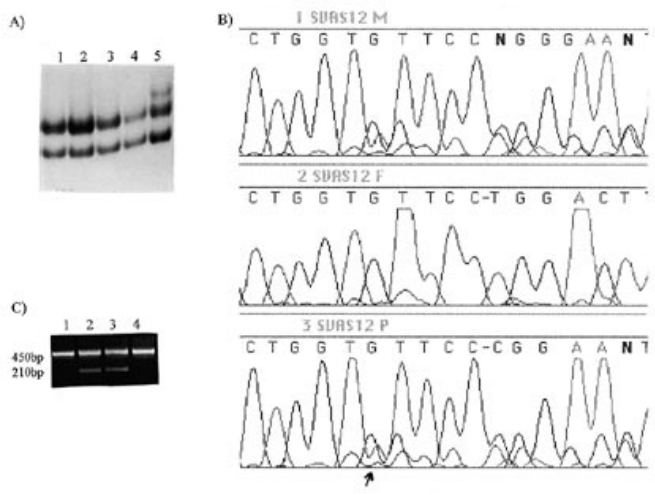

Figure 2. Mutation analysis of patient SVAS12. (**A**) Heteroduplex analysis of exon 26 PCR product, showing a shift in lane 5. (**B**) Double strand sequence analysis shows the proband (bottom) and his mother (top) are heterozygous for a T insertion, resulting in a frameshift. The father (middle) has two normal alleles. (**C**) Confirmation of the mutation by ARMS PCR analysis. The allele-specific ARMS primer allows selective amplification of the mutant allele only in the proband and his mother, giving a 210 bp product. An internal control giving a 450 bp band was included to ensure that amplification had taken place in each sample. Lane 1: father; lane 2: proband; lane 3: mother; lane 4: control.

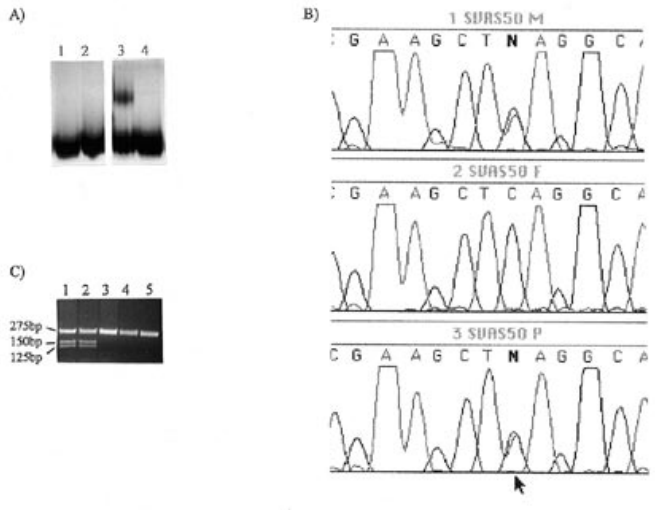

Figure 3. Mutation analysis of patient SVAS50. (**A**) Heteroduplex analysis of exon 1 showing a shift in lane 3. Lanes 1, 2, 4: controls; lane 3: patient SVAS50. (**B**) Double strand sequence analysis shows a C–T base substitution (N = C or T) in one allele of the proband (bottom) and his mother (top). The father (middle) has two normal alleles. (**C**) Confirmation of the mutation by *Hin*dIII digestion. The C–T base substitution creates a *Hin*dIII site that cleaves the 275 bp product into fragments of 150 and 125 bp that are present only in the proband and his mother. Lane 1: mother; lane 2: proband; lane 3: father; lanes 4, 5: controls.

The PCR product from the normal allele is not digested (275 bp), whereas the mutant allele gives two restriction fragments of 150 bp and 125 bp (Fig. 3). Restriction digest analysis and sequencing showed that the Q442X mutation was also present in the mother's DNA (Fig. 3).

DISCUSSION

Elastin is an important structural component of the aorta and pulmonary arteries. The walls of these large elastic vessels are primarily composed of alternating layers of smooth muscle and elastic fibres. Two theories explaining the molecular pathology of SVAS can be proposed: the defects in elastin could be qualitative or quantitative. Evidence favouring a quantitative mechanism rather than production of abnormal elastin, as the pathogenic mechanism in SVAS includes the fact that Williams syndrome (WS) patients are hemizygous for elastin because of a chromosomal microdeletion, and these patients are at high risk of SVAS. Also elastin mutations reported so far in patients with isolated SVAS have been disruptions or large deletions. Curran *et al.* (9) reported a family segregating a balanced translocation t(6:7)(p21.1;q11.23) with a breakpoint in intron 27 of the *ELN* gene. Ewart *et al.* (11) described a 100 kb deletion in an SVAS patient, which deleted all 3′ *ELN* sequences from exon 28. Olson *et al.* (12) reported a patient with severe SVAS who had a 30 kb intragenic deletion that removed exons 2–27 of the elastin gene whilst maintaining the 3′ terminus intact. Our two SVAS patients have point mutations that are predicted to truncate the transcript in exon 21 or exon 26. *ELN* transcripts lacking the 3′ terminus may be unstable or poorly translated. High sequence homology (80%) in the 3′ untranslated region in different species suggests that it has an important conserved function and may play a role in stabilising the mature mRNA or in modulating translation(20). Finally, variable expressivity and reduced penetrance of SVAS is seen both in Williams syndrome and familial isolated SVAS. SVAS varies from subtle cardiac abnormalities to severe stenosis of multiple arteries, although there is no evidence of locus heterogeneity. In our two SVAS families with point mutations, each mutation manifests as severe SVAS in the proband, but as mild cardiac features or non-penetrance in the mothers. Such variability is typical of phenotypes produced by haploinsufficiency, where genetic background is expected to have a major modifying effect.

The alternative hypothesis is that a dominant negative elastin mutation could result in SVAS. Research into the assembly of elastic fibres suggests that the C-terminus of tropoelastin mediates elastin polymerisation through interaction with microfibril-associated glycoproteins (MAGP) (21). If the mutant elastin genes described above are expressed, truncated proteins would probably be produced that would lack several important features. These include consensus sites for desmosine cross-linking, exon 36 (which contains important functional domains), and also the two highly conserved cysteine residues in exon 34 thought to be important for interaction with fibrillin in arrays of microfibrils, as well as a MAGP binding domain. Truncated proteins in which some but not all domains critical for intermolecular interaction are absent may disrupt post-translational processing and consequently, the development of elastic fibres.

It is possible that SVAS arises as a result of both mechanisms, depending on the particular mutation involved. Haploinsufficiency of elastin, where a half dose of normal elastin is being produced, or abnormal elastic fibres arising from dominant negative elastin mutations, could both manifest as SVAS. It is intriguing that WS patients show a wider range of connective tissue phenotypes than SVAS patients—whether this is caused by deletion or silencing of other adjacent genes remains to be seen.

However, no obvious phenotype–genotype correlation has emerged in SVAS.

Elucidating the molecular pathogenesis of SVAS may also have implications for treatment. Pre-natal diagnosis can be offered to families with autosomal dominant SVAS to allow early diagnosis and treatment. At present vascular surgery is the only treatment for SVAS; however, it has been suggested that early intervention with drug treatment to lower the heart rate and blood pressure may slow progression of the disease (9). The use of elastase inhibitors in preventing pulmonary hypertension and associated pulmonary arterial abnormalities is also being investigated (23).

Defining the elastin gene structure has allowed us to identify the first point mutations that cause SVAS and will enable us to carry out mutation screening on a larger scale. Continued mutation analysis in SVAS and WS patients may help distinguish the vascular pathology of these conditions, and will allow other conditions to be investigated for involvement of elastin mutations.

MATERIALS AND METHODS

Exon–intron structure of ELN

A 125 kb BAC clone containing the elastin gene was isolated from a BAC library (Genosys) by PCR screening with primers P1–P2 and P3–P4, designed from the *ELN* cDNA sequence:

Primer P1: 5′ CCG GGA TAA AAC GAG GTG CGG GAG
Primer P2: 5′ TCC AGG CCG AGA GGG GTG GAG GAT
Primer P3: 5′ AGC CGA AAC TGA GAG GGG CCG GAC
Primer P4: 5′ TCA TTT TCT CTT CCG GCC ACA AGC

FISH analysis showed that the BAC hybridised to 7q11.2 and that it was not chimaeric. Vectorette libraries were constructed from *Eco*RI, *Eco*RV, *Rsa*I and *Pvu*II-digested BAC DNA and subjected to PCR amplification using a vectorette specific primer and one cDNA primer as described by Riley *et al.* (15). Column-purified PCR products were sequenced by direct double-strand fluorescent cycle sequencing using an ABI 373 sequencer. Intron PCR was carried out using the BCL XL Expand system according to the manufacturer's instructions.

Elastin FISH and microsatellite analysis

Elastin FISH was carried out using the Oncor WSCR probe according to manufacturer's instructions. Intron 18 microsatellite analysis was carried out by PCR amplification of genomic DNA using the following primers:

ELN intron 18F: 5′ ATG AGA CGT GGT CAA GGG TAT
ELN intron 18R: 5′ GGG ATC CCA GGT GCT GCG GTT

All amplifications were carried out using 100 ng of genomic DNA and 10 pmol of each primer in 20 µl reaction volumes. Cycle conditions were: 95°C for 2 min, then 27 cycles of: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension step of 5 min at 72°C. PCR products were electrophoresed on 8% polyacrylamide gels (acrylamide:*N*,*N*′ bis-acrylamide 19:1) for 3 h at 300 V then visualised by silver staining.

Mutation detection and sequencing

Exons 21 and 26 of the ELN gene were PCR amplified using the primers listed below and amplification conditions as described above. Mutations were detected by a combination of SSCP and heteroduplex analysis (18). Briefly, PCR products were run on 1 mm thick non-denaturing 8% polyacrylamide gels (acrylamide:*N*,*N*′ bisacrylamide 49:1) at 4°C overnight at a constant 350 V. Products were detected by silver staining. Mutations were initially characterised by direct double-strand cycle sequencing of column-purified PCR products in both orientations with a matched control on a fluorescent sequencer (ABI 373).

ELN X21F : 5′ GAG GTC GTA TCC ATG CCT TAC AG
ELN X21R: 5′ TCC AGG CCA TTT CAG TCC TGG AG
ELN X26F: 5′ GGC ATG CTC CCT GCC TGC TGT CG
ELN X26R: 5′ CCC AGA TGC TTA GGA GAA CCT AA

Confirmation of mutations

For confirmation of the exon 21 mutation, *Hin*dIII digestion of PCR products was carried out according to manufacturer's instructions (Gibco-BRL). The restriction fragments were separated on a 2% agarose gel and visualised by ethidium bromide staining. For confirmation of the exon 26 mutation, ARMS PCR (19) was carried out using the ELN X26R primer and a primer designed to amplify the mutant allele only:

ELN X26R: 5′ CCC AGA TGC TTA GGA GAA CCT AA
ELN X26F ARMS M: 5′ TGG ACT TGG AGT TGG TGC TGA TT

No amplification is obtained in the absence of the mutation, so intron 26 of the elastin gene was used as an internal control for amplification, which gave a 450 bp product. PCR was carried out as above. Cycle conditions were: 95°C for 2 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension step of 5 min at 72°C. The products were separated on a 2% agarose gel.

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