

## ARTICLES

# Novel mutations in Rsk-2, the gene for Coffin-Lowry syndrome (CLS)

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**Coffin-Lowry syndrome (CLS) is an X-linked disorder characterized by facial dysmorphism, digit abnormalities and severe psychomotor retardation. CLS had previously been mapped to Xp22.2. Recently, mutations in the ribosomal S6 kinase (Rsk-2) gene were shown to be associated with CLS. We have tested five unrelated individuals with CLS for mutations in nine exons of Rsk-2 using Single Strand Conformation Polymorphism (SSCP) analysis. Two patients had the same missense mutation (C340T), which causes an arginine to tryptophan change (R114W). This mutation falls just outside the N-terminal ATP-binding site in a highly conserved region of the protein and may lead to structural changes since tryptophan has an aromatic side chain whereas arginine is a 5 carbon basic amino acid. The third patient also had a missense mutation (G2186A) resulting in an arginine to glutamine change (R729Q). The fourth patient had a 2bp deletion (AG) of bases 451 and 452. This creates a frameshift that results in a stop codon 25 amino acids downstream, thereby producing a truncated protein. This deletion also falls within the highly conserved amino-catalytic domain of the protein. The fifth patient has a nonsense mutation (C2065T) which results in a premature stop codon, thereby producing a truncated protein. These mutations further confirm Rsk-2 as the gene involved in CLS and may help in understanding the structure and function of the protein.**

**Keywords:** Coffin-Lowry; Rsk-2; SSCP; mental retardation; mutation

## Introduction

Coffin-Lowry syndrome is an X-linked disorder characterized as an inherited facio-digital mental retardation syndrome.<sup>1</sup> Patients diagnosed with CLS exhibit skeletal anomalies with severe psychomotor retardation and their hands appear to be bulbous with tapering

fingers. Other clinical features include patulous lips, larger mouth, anteverted nares, hypertelorism, and prominent frontal region.<sup>2</sup> Affected males exhibit severe mental retardation along with characteristic dysmorphic features, whereas in females the phenotypic spectrum covers the entire spectrum including normal appearance or minor dysmorphic features, or the complete syndrome as seen in the male. Linkage studies mapped the CLS locus to Xp22.2, between DXS365 and DXS7161, an interval of approximately 3 cM.<sup>3–5</sup> Recently Trivier *et al*<sup>6</sup> showed that mutations in the Rsk-2 gene are associated with CLS. The Rsk-2 gene is a member of the RSK (ribosomal S6 kinase)

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Received 28 January 1998; revised 6 May 1998; accepted 29 May 1998

**Table 1** Primers and conditions used for genomic amplification of nine Rsk-2 exons

Exon coverage	Primer name/sequence	Annealing temp	Product length (bp)
nt 326–406	VH236: AGTGGATATTACAATGTAGCA VH237: GGGAGACGGCTCATACTTA	55°C	140
nt 407–486	VG18: TATGAATGACCTAATGTAAACC VG19: CAGGATGCATGTAAATAGACT	55°C	190
nt 487–593	VI242: ACAGACACAAAAAGAAAATAAAT VN206: ATCATATTACATTGTATTCAACT	50°C	200
nt 1000–1102	VP47: GTATAGAATGAAAACCTTGCTTG VP126: TATTTGTTGTCTTATATTTGGAT	58°C	200
nt 1354–1443	VS157: TGTATAGTTGATGAGGTTT VH240: AGACAACTGATTCAAATGA	47°C	170
nt 1766–1841	VH244: CTAATTTGCACTTTTTTCTA VH245: AATGNTTAGGTGGCTTAGA	54°C	130
nt 1842–1959	VH242: TTTGATAGGAAGTGATACT VH243: GAGTACTTTTGAGATTACC	47°C	210
nt 1960–2100	WL166: AAGAGCCTAGAAAAAGC WL168: TGGAGGACCTGTGGAAAAC	55°C	200
nt 2100–2259	WL171: TGTGTACGTGTGACTATCCATT W191: GTGTGCTTGCAGGTGTCTCTC	55°C	200

family that consists of growth factor regulated serine-threonine kinases also known as p90<sup>msk</sup>.<sup>7</sup> The Rsk-2 cDNA has an open reading frame of 2220 bp and it encodes a protein of 740 amino acids.<sup>8</sup> In humans, the RSK family is comprised of three isoforms, Rsk-1, Rsk-2 and Rsk-3,<sup>7</sup> which encode proteins that contain two non-identical kinase catalytic domains.<sup>9</sup>

In their report, Trivier *et al*<sup>6</sup> reported a total of six mutations in the Rsk-2 gene: two missense mutations, one nonsense mutation, two deletions and one insertion. Three of the mutations were in the N-terminal kinase catalytic domain and the other three were in the C-terminal kinase catalytic domain. Four of the six mutations resulted in the truncation of the Rsk-2 protein.

In this study we have tested five unrelated patients with CLS for mutations in the Rsk-2 gene. Three patients were familial and two were sporadic cases. We have identified two missense mutations, one nonsense mutation and a 2-base pair deletion. One of the missense mutation (C340T) is present in two of the patients. Thus, this study further confirms Rsk-2 as the gene involved in CLS.

## Materials and Methods

### Patients

Five males, two Caucasian and three African-American, carrying the diagnosis of Coffin-Lowry (CLS), were available for analysis. Three of the probands had at least a brother who also carried the diagnosis of CLS. The fourth male had a

family history of mental retardation in male relatives and his mother and aunt had been assessed as having intellectual impairment. The remaining male had no family history of mental retardation and both parents were reported to have been normal. All of the probands had clinical findings consistent with CLS: large, soft hands with tapering fingers, severe to moderate mental retardation, short stature ( 5th centile), weight below the 5th centile, microcephaly, telecanthus or hypertelorism, prominent eyes. The Caucasian probands had large mouths and prominent lower lips. For the African-American probands this was difficult to determine because of racial background.

### PCR Analysis

Genomic DNA was isolated from lymphocytes using a high salt procedure.<sup>10</sup> Purified DNA was diluted with TE (10 mM Tris, 1 mM EDTA pH 8.0) to a final concentration of 105 µg/ml and stored at 4°C. Hundred nanograms of genomic DNA were amplified in a total volume of 20 µl containing 1X PCR reaction buffer (10 mM Tris, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, pH 8.3) with 1 µM of each primer, 50 µM of dNTPs, 1 unit Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA), and 0.1 µg of TaqStart antibody (Clontech, Palo Alto, CA, USA).<sup>11</sup> Amplification was done in a 9600 Thermocycler (Perkin Elmer/Cetus, Foster City, CA, USA). The information on the PCR primers and their optimal annealing conditions are listed in Table 1. The primers were designed using sequence flanking the particular exon to insure detection of any alteration in the splice junction. All primers were synthesized and FAM labeled with FlouerePrime (Pharmacia Biotech, Piscataway, NJ, USA), using an Oligo 1000 DNA synthesizer (Beckman, Fullerton, CA, USA) and were desalted over a NAP10 column (Pharmacia Biotech, Piscataway, NJ, USA). Ten microlitres of the amplified products were checked on a 2% agarose gel to confirm product amplification.

**Table 2** Rsk-2 mutations in 5 CLS patients

Patient	Mutation	Predicted protein sequence*
2598 and CMS0976	C340T	GACCGAGTTCGGACAAAA D R V W T K
Control		GACCGAGTTTGGACAAAA D R V R T K
CMS0429	G2186A	ACTCTTGCTCAGCAGAGAGGT T L A Q Q R G
Control		ACTCTTGCTCAGCGGAGAGGT T L A Q R R G
4534	C2065T	CTGCCACAATACTAACTAAAC L P Q Y * L N
Control		CTGCCACAATACCACTAAAC L P Q Y Q L N
2423	451delAG	TTGGATTTTCTC—GGGAGGAGATTTGTTT.....CTTGGCTGA L D F L G R R F V L G *
Control		TTGGATTTTCTCAGGGGAGGAGATTTGTTT.....ACTTGGCTG L D F L R G G D L F Y L A

\*Predicted amino acid sequence is compared to the normal sequence (listed as control).

### SSCP and Sequence Analysis

At the time of this study, the exon/intron boundary of only 9 exons had been identified in the Rsk-2 gene. SSCP analysis of 7 of the exons was done on the Automated Laser Fluorescence (ALF) DNA sequencer (Pharmacia Biotech, Piscataway, NJ, USA). Products that showed an altered pattern on the SSCP gel were subcloned. As a control, a corresponding PCR product amplified from a normal individual was also subcloned. The cloned DNA was sequenced using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Arlington Heights, IL, USA), FAM-labeled M13 (-40) forward and reverse primers (Pharmacia Biotech, Piscataway, NJ, USA). The sequence was analyzed on the ALF DNA sequencer.

SSCP analysis was done manually for the 2 exons at the 3' end of the gene. PCR amplification was done in the presence of 1 pmol (3mCi) of <sup>32</sup>P dCTP, after which aliquots of 4 µl were denatured for 5 min at 90°C and loaded on a 10% glycerol, 6% polyacrylamide gel (acryl-bisacryl, 49:1). Electrophoresis was run at 4 W for 10–12 hrs in the cold room. The gel was dried and exposed overnight.

### Mutation Analysis

The point mutations C340T and C2065T did not create or remove any restriction site. Therefore a modified primer was designed to create or eliminate a restriction site near the mutation of interest.<sup>12</sup> For the C340T mutation, primer VH236A (5'CTTTTATAGTTCGAGACCGAGTC 3'), was designed to introduce a C at position 339, next to the C to T alteration. This primer creates a MspI site in the wild type. Primer VH236A, in combination with VH237, yields a product of 110 bp, which upon digestion with MspI results in two fragments (23 bp and 87 bp). The fragments were resolved by electrophoresis on a 8% acrylamide/15% glycerol mini gel (Hofer, San Francisco, CA, USA).

For the C2065T mutation, primer Ex21F (5'GGGAC-CAACTGCCACAATAT 3') was used to introduce a T at

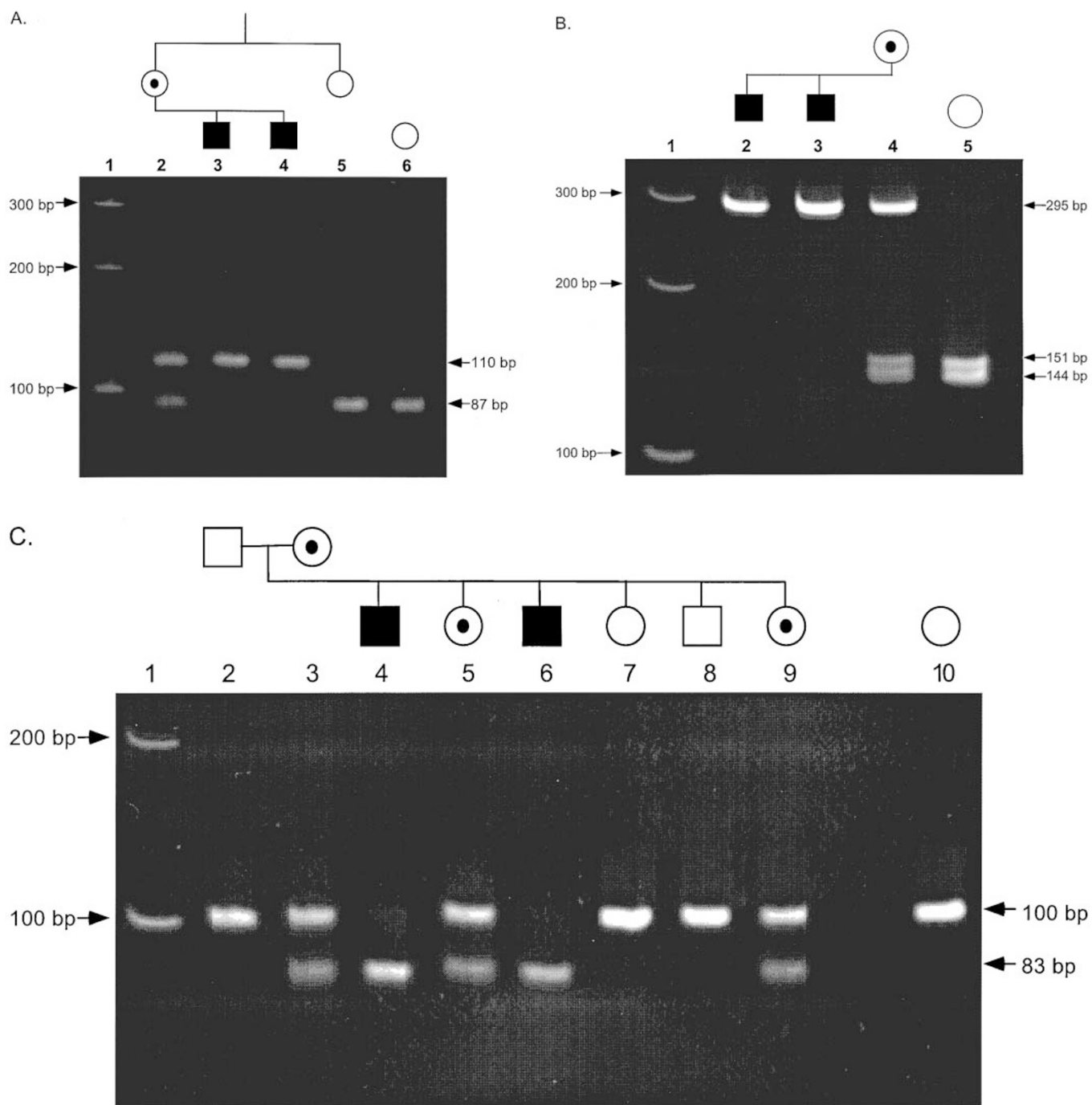
position 2064. This primer creates a SspI site in the patient. Primer Ex21F, in combination with WL168, amplified a product of 100 bp, which when digested with SspI gives two fragments (83 bp and 17 bp) in the patient.

The point mutation G2186A resulted in the loss of MspAII site in the patient which made it possible to test the mutation in the family and in normal chromosomes.

## Results

Two of the patients (2598 and CMS0976) showed the same abnormal SSCP pattern in the exon defined by nt 326–406 (Table 1). Sequence analysis revealed a C to T mutation (C340T) in both patients, resulting in an arginine to tryptophan change at position 114 (R114W) in the protein (Table 2). The C340T mutation did not create or remove a restriction site. Therefore an alternative primer, VH236A, was used in combination with VH237 (see Material and Methods) to yield a product of 110 bp. Utilization of primer VH236A created a novel MspI site in the normal DNA sequence. The family of patient 2598 was tested and the C to T mutation was shown to segregate with CLS (Figure 1a). As patients 2598 and CMS0976 are African-American, 100 X chromosomes from African-American individuals were analyzed for the mutation. The C340T alteration was not observed in this sample indicating it is not a rare polymorphism (data not shown).

Patient CMS0429 showed an altered SSCP pattern in the exon containing the nucleotide region 2100–2259



**Figure 1** Segregation of mutations in *Rsk-2* in the three familial cases. **a:** Segregation of the C340T mutation in the family of proband 2598. The PCR product created using primers VH236A and VH237 was digested with *MspI* and resolved on a 8% acrylamide gel. Lane 1: 100 bp ladder (GIBCO BRL). Lane 2: Mother. Lanes 3 and 4: The proband 2598 and his affected brother respectively. Lane 5: Aunt of the proband and his brother. Lane 6: Control individual. **b:** Segregation of the G2186A mutation in the family of proband CMS0429. The PCR product created using primers WL171 and W191 was digested with *MspAII* and resolved on a 8% acrylamide gel. Lane 1: 100 bp ladder. Lane 2 and 3: The proband CMS0429 and his affected brother respectively. Lane 4: Mother. Lane 5: Control individual. **c:** Segregation of the C2065T mutation in the family of proband 4534. The PCR product generated by using primers Ex21F and WL168 was digested with *SspI* and resolved on a 8% acrylamide gel. Lane 1: 100 bp ladder. Lane 2: Father. Lane 3: Mother. Lanes 4 and 6: Proband 4534 and his affected brother respectively. Lanes 5 and 9: Sisters, who appear to be carriers of the mutation. Lane 7: Sister, who is normal. Lane 8: Normal brother. Lane 10: Control individual

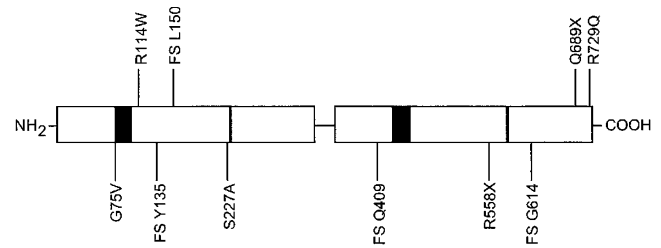
(Table 1). Sequence analysis of patient CMS0429 revealed a G to A (G2186A), which caused a missense mutation (R729Q) (Table 2). This mutation resulted in the loss of an MspA1I site in the patient, which made it possible to test the mutation in the family and population. The affected brother and mother of the patient CMS0429 were tested and the G2186A alteration was shown to segregate with the disease (Figure 1b). Analysis of 100 X chromosomes did not detect any G2186A mutation ruling out a rare polymorphism (data not shown).

Patient 4534 showed an abnormal SSCP pattern in the exon defined by nt 1960–2100 (Table 1). Sequence analysis of the patient showed that he was carrying a C to T (C2065T) mutation that gives rise to a premature stop codon (Q689X), and a truncated protein lacking the last 51 amino acids of the Rsk-2 gene (Table 2). The C2065T mutation did not create or destroy any restriction sites, therefore a new primer Ex21F was designed (see Material and Methods) which created an SspI site in the patient. The family of the proband 4534 was tested for the C2065T mutation which was observed to segregate with the disease (Figure 1c). Two of the proband's sisters were found to be carriers of the mutant allele as a result of this test. Since this is an African-American family, 100 X chromosomes from African-American individuals were tested for the alteration. The C2065T mutation was not detected in this set of chromosomes which indicated that it is not a rare polymorphism (data not shown).

The fifth patient (2423) exhibited an altered SSCP pattern in the exon containing the nucleotide region 407–486 (Table 1). Sequence analysis found a 2 bp deletion (AG) of bases 451 and 452 (Table 2). This deletion creates a significant frame shift and causes a premature termination 75 bp downstream, thereby producing a severely truncated protein (Table 2). To test this deletion, PCR products from 100 normal chromosomes were separated on a 6% Long Ranger and 5.6 M Urea gel using the ALF DNA sequencer. Only the patient 2423 showed a shift of 2 bp (data not shown).

## Discussion

Trivier *et al*<sup>6</sup> identified the Rsk-2 gene as the candidate gene for CLS based on mutational analysis. In all, these authors found six mutations. Four of these mutations – two deletions, one insertion and one nonsense mutation – resulted in the truncation of the protein. The other two alterations were missense mutations involving



**Figure 2** Schematic representation of the protein encoded by the Rsk-2 gene showing the distribution of mutations. The open boxes represent the two non-identical kinase catalytic domains, containing the ATP binding sites (black boxes) and the APE sites (vertical line). Mutations described in the present study are drawn above the schematic representation, and mutations reported previously are drawn below. Frameshift mutations are designated as FS, followed by the abbreviation and position of the amino acid at which the sequence begins to change

glycine and serine residues at positions 75 and 227 respectively (Figure 2). The glycine residue at position 75 is located within the highly conserved ATP-binding site, and the serine residue at position 227 is the putative phosphorylation site of the kinase domain. Thus both mutations likely affected the activity N-terminal catalytic domain. Trivier *et al*<sup>6</sup> were able to confirm this for the S227A mutation by showing a reduction in S6 kinase activity. This finding demonstrated that the CLS phenotype appeared to be linked to the loss of Rsk-2 biological function.

The mutations found in the Rsk-2 gene in our five Coffin-Lowry patients further confirms it as the gene associated with CLS. The C340T (R114W) missense mutation was found in two unrelated patients of the same racial background (Table 2). At present, this is the first instance of the same mutation occurring in more than one patient with CLS. The mutation is in a conserved region which is 12 amino acids downstream from the first ATP binding site of the N-terminal kinase catalytic domain. Protein database comparison of this region showed that it is highly conserved in other organisms such as *Xenopus laevis*, *Mus musculus*, and *Gallus gallus*.<sup>13,14</sup> Since the mutation replaces an arginine (positively charged) with a tryptophan (polar), there could be a conformational change in the protein due to the structure and charge of the substituted amino acid. This structural change may effect the binding of ATP, thereby reducing the autophosphorylation, which is predominantly mediated by the N-terminal kinase catalytic domain.<sup>15</sup> The other missense mutation (R729Q), in patient CMS0429, involves the substitution of an uncharged glutamine residue for a basic arginine residue. As this arginine is highly

conserved and occurs in a region suspected to interact with mitogen-activated protein kinases (MAPKs),<sup>16</sup> it is very likely the alteration in charge adversely affects this interaction.

The remaining two mutations lead to a truncation of the Rsk-2 protein. The nonsense mutation (Q689X) in patient 4534 results in a premature stop codon (TAA) which eliminates the last 51 amino acids of the protein. The 2 bp deletion of bases 451 and 452 in patient 2423 causes a significant frameshift resulting in a TGA stop codon 75 bp downstream (Table 2). This results in a truncated protein that lacks the major portion of the N-terminal kinase domain and the entire C-terminal kinase domain.

Of the 10 mutations found by us and Trivier *et al*,<sup>6</sup> six result in the creation of a truncation of the Rsk-2 protein. Although these numbers are small, the results indicate that the protein truncation test (PTT) may be useful for mutation detection for CLS.<sup>17</sup>

The C-terminal kinase domain of Rsk-2 interacts with MAPKs, which has been shown to phosphorylate and further stimulate the autophosphorylation of the Rsk-2 C-terminal domain. Once the C-terminal kinase domain is phosphorylated, it is able to phosphorylate and activate the N-terminal kinase domain. Therefore both the kinase domains are required for the maximal activity of the protein.<sup>15</sup> The patient with the 451delAG deletion, which truncates the protein in the N-terminal kinase catalytic domain, has a more severe phenotype than the patient with Q689X, the nonsense mutation which truncates the protein at the C-terminal end (Figure 2). This patient has been non-ambulatory since the age of 11, a rare occurrence in CLS patients. This would indicate that loss of function of both kinase domains is more detrimental than the loss of the C-terminal kinase activity as expected based on our knowledge of members of the RSK family.<sup>15</sup> Similar phenotype-genotype correlations will now be possible for other CLS patients and may address the variable degree of mental retardation which exists in CLS patients.<sup>6</sup>

## Acknowledgements

We thank Dan Riconda, MS and Drs David Flannery, Cindy Powell, Richard Schroer and Laurie Seaver for contributing the patients. Dr Bernhard Hane assisted in running the SSCP analysis and the sequencing gels on the ALF. This work was supported, in part, by grants from NICHD (2RO1HD26202) and The Fullerton Foundation (97-4) to CES, and a grant from the South Carolina Department of Disabilities and

Special Needs (SCDDSN). We also thank the Commission of the European Communities and the Association Française contre les Myopathies for their support. CL was supported by the Furman Advantage Internship Program.

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