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Unusual Splice-Site Mutations in the *RSK2* Gene and Suggestion of Genetic Heterogeneity in Coffin-Lowry Syndrome

Maria Zeniou,[1] Solange Pannetier,[1] Jean-Pierre Fryns,[2] and André Hanauer[1]

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique/INSERM/Université Louis Pasteur, Illkirch, France; and [2]Clinical Genetics Unit, Center for Human Genetics, University of Leuven, Leuven, Belgium

Coffin-Lowry syndrome (CLS) is a syndromic form of X-linked mental retardation that is characterized, in male patients, by psychomotor and growth retardation and various skeletal anomalies. Typical facial changes and specific clinical and radiological hand aspects exhibited by patients are essential clues for the diagnosis. CLS is caused by mutations in a gene that is located in Xp22.2 and that encodes RSK2, a growth-factor–regulated protein kinase. *RSK2* mutations are extremely heterogeneous and lead to premature termination of translation and/or loss of phosphotransferase activity. Surprisingly, among a series of 250 patients screened by single-strand conformation polymorphism (SSCP) analysis, in whom a clinical diagnosis of CLS was made, no mutations were detected in 66% (165) of the patients. To determine what proportion of these latter patients have a *RSK2* mutation that has not been detected and what proportion have different disorders that are phenotypically similar to CLS, we have, in the present article, investigated, by western blot analysis and in vitro kinase assay, cell lines from 26 patients in whom no mutation was previously identified by SSCP analysis. This approach allowed us to identify seven novel *RSK2* mutations: two changes in the coding sequence of *RSK2*, one intragenic deletion, and four unusual intronic nucleotide substitutions that do not affect the consensus GT or AG splice sites. We have also determined the nucleotide sequence of the promoter region of the *RSK2* gene, and we have screened it for mutations. No disease-causing nucleotide change was identified, suggesting that mutations affecting the promoter region are unlikely to account for a large number of patients with CLS. Finally, our results provide evidence that some patients have a disease that is phenotypically very similar to CLS, which is not caused by *RSK2* defects. This suggests that there are defects in either additional genes or combinations of genes that may result in a CLS-like phenotype.

Introduction

Coffin-Lowry syndrome (CLS [MIM #303600]) is a syndromic form of X-linked mental retardation that initially was independently described by Coffin et al. (1966) and Lowry et al. (1971) and was definitively distinguished by Temtamy et al. (1975). Cardinal features of CLS are growth and psychomotor retardation, characteristic facial and digital abnormalities, and progressive skeletal alterations. CLS occurs in all ethnic groups.

Adult male patients typically are of short stature and exhibit a characteristic coarse face with a prominent forehead, orbital hypertelorism, downward-slanting palpebral fissures, epicanthic folds, prominent ears, thick lips, a thick nasal septum with anteverted nares, and irregular or missing teeth. Their large and soft hands with lax skin and tapering fingers are usually a strong diagnostic feature. The most frequent skeletal involvements are delayed bone development, spinal kyphosis/scoliosis, and pectus carinatum or excavatum. Cognitive function may be variably impaired, but most males are severely affected. Development of speech is always involved to variable degrees. Features such as microcephaly, ventricular dilatation, sensorineural deafness, seizures, and cardiac defects may be present but are less frequent. In addition, connective-tissue abnormalities, such as inguinal hernia, may be present. A majority of carrier females have only minimal findings (e.g., mild facial coarsening, tapering fingers, and obesity) (Hunter et al. 1982; Gilgenkrantz et al. 1988; Young 1988; Plomp et al. 1995).

Although the clinical diagnosis of CLS is based solely on the presence of the characteristic physical findings, clinicians trained in genetic disorders can often make the diagnosis in adult patients. Recognition of CLS in young children is more difficult, since physical characteristics are generally mild and less specific. Affected newborn males usually show only hypotonia and hyperlaxity of joints. Retardation of growth and psychomotor development appear gradually in the first years of life. Facial coarsening, which is mild and hard to

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Address for correspondence and reprints: Dr. André Hanauer, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Boîte Postale 10142, 67404 Illkirch Cedex, Communauté Urbaine de Strasbourg, France. E-mail: andre@titus.u-strasbg.fr

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detect in infancy, becomes progressively pronounced and characteristic only in late childhood or adolescence (Wilson and Kelly 1981; Plomp et al. 1995).

We have demonstrated that CLS is caused by loss-of-function mutations in the gene that encodes RSK2 (ribosomal S6 kinase 2), a serine/threonine kinase from the family of MAPK (mitogen-activated protein kinase) activated protein kinases (Trivier et al. 1996). The coding region of the *RSK2* gene, which maps to Xp22.2, is split into 22 exons (Jacquot et al. 1998) and encodes a 740-amino-acid protein. We have previously shown that this gene is subject to strong allelic heterogeneity, with >70 different mutations so far identified (see Coffin Lowry Syndrome Mutations Data Base). These changes are scattered, without any clustering, throughout the coding region, and most are family specific; they include single-base-pair substitutions that cause amino acid changes, premature stop codons, splice defects, and small insertions and deletions that result in frameshifts (Delaunoy et al. 2001). Unexpectedly, mutations have been identified in only 34% of individuals with a clinical diagnosis of CLS who were referred for mutation screening. There are several possible explanations for the failure to detect mutations in such a high proportion (66%) of patients, and it is likely that more than one of these explanations needs to be invoked to explain the discrepancy. It may be related to the known limitations of SSCP analysis, which was almost exclusively used in the mutation screening (Orita et al. 1989). The *RSK2* gene may also contain mutations in sequences, such as the promoter region or introns, that have not been screened. In addition, it is possible that some of the patients were atypical and that a misdiagnosis thus had been made. Nevertheless, detailed clinical records could be obtained for a number of individuals, providing evidence that some patients without an *RSK2* mutation do indeed exhibit a typical CLS phenotype. Therefore, a further possibility is that mutations in a gene (or genes) distinct from *RSK2* may give rise to a disorder (or disorders) phenotypically very similar to CLS.

Mutations in the *RSK2* gene that have so far been found result in truncated proteins, not detectable on western blot with the currently available antibodies, and/or a dramatically reduced RSK2 phosphotransferase activity, when tested in an in vitro S6 kinase assay (Trivier et al. 1996; Merienne et al. 1998). Together, these two assays are expected to detect all classes of mutations and, in particular, all those classes that either are not detectable by SSCP analysis or may affect the level of transcription. Therefore, in the present article, we have further investigated, by western blot analysis and in vitro kinase assay, cell lines from 26 unrelated patients in whom no mutation was previously identified by SSCP analysis. Although some of these patients exhibited additional features not commonly associated with CLS, all had physical findings that were characteristic of CLS. This approach allowed us to identify seven patients who carry RSK2 mutant proteins. The nature of the mutations has been determined. Additionally, we have determined the nucleotide sequence of the promoter region of the *RSK2* gene, and we have screened it for mutations. No disease-causing nucleotide change was identified. Importantly, our results provide evidence that some patients have a disease that is phenotypically very similar to CLS but is not caused by *RSK2* defects. This suggests that defects in other genes may result in a CLS-like phenotype.

Patients and Methods

Patients

Twenty-six unrelated patients with the diagnosis of CLS and from whom a cell line was available were included in this study. They were referred by 18 different European and American pediatricians or clinical geneticists, and no mutations were detected by SSCP analysis, as described elsewhere (Jacquot et al. 1998; Delaunoy et al. 2001). A detailed clinical questionnaire (completed by the clinicians), for each patient, and photographs, for two-thirds of the patients, were available for review. All of these patients exhibited clinical features highly suggestive of CLS (table 1).

Cell Culture

Patient lymphoblastoid cell lines were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum. Fibroblast cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Western Blot Analysis

Pellets from confluent lymphoblastoid and fibroblast cell lines were washed once with ice-cold PBS and were resuspended in 500 μl cold NP40 lysis buffer (50 mM Tris-HCl, with pH 7.4; 1% NP40; 0.5% Triton 1-100; 150 mM NaCl; 1 mM EDTA; 1 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride; and 1× protease inhibitor cocktail). After 30 min on ice, the lysates were centrifuged for 15 min at 13,000 rpm at 4°C. The pellets were discarded, and the protein content of the supernatants was determined using the BIO-RAD protein assay (BioRad). The cell lysates (100 μg total protein) were resolved by standard SDS/6% PAGE. After 1 h transfer to Protran nitrocellulose membranes (Schleicher and Schuell), the RSK2 protein was detected with the mouse monoclonal E1 antibody (Santa Cruz Biotechnology), which was raised against the C-terminus of the human RSK2 protein. The amount of ERK2 protein, used as an internal control, was determined

Table 1

Clinical Data

Characteristic	Data for Patient																									
	AD57	CV59	EZ48	DZ75	GW55	GW87	DZ91	DX43	DX55	DX86	DZ32	DZ36	DZ37	EZ14	FP41	FP67	GM10	GM48	GM49	GM98	GW11	GW15	GW24	GW44	GW85	MH55
RSK2 mutation	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Age at examination (years)	21	10	7	7	12	30	5	20	35	29	38	40	18	9	3	1	6	9	10	13	13	11	12	1	28	10
Neurological:																										
Mental retardation	S	Mi	Mo	S	S	S	Mo	Mo	S	S	Mo	S	S	S	S	S	S	S	S	S	S	S	Mi	S	S	Mo
Developmental delay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Speech delay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hypotonia	+	+	+	+	+	…	+	+	+	+	…	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
Ventricular dilatation	…	…	−	−	…	−	…	…	…	+	…	…	…	…	…	−	…	…	…	…	−	−	…	−	−	…
Sensorineural hearing deficit	−	+	−	−	+	…	…	−	…	−	+	−	+	…	+	+	…	−	−	+	−	−	+	−	−	−
Seizure	+	−	+	…	…	…	…	−	+	−	+	+	+	…	+	…	…	+	−	…	…	…	+	…	+	−
Drop attacks	+	−	+	+	…	+	…	−	+	…	…	−	+	…	−	…	…	…	…	…	+	+	+	−	…	…
Growth:																										
Small stature	+	+	+	…	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	…	+	+	+
Stooped posture	…	+	…	…	…	+	…	−	…	+	+	+	+	…	…	…	+	+	+	…	+	+	…	+	…	…
Facial features:																										
Microcephaly	…	+	…	…	…	…	…	−	…	−	…	−	…	…	…	+	…	…	…	+	+	+	…	−	…	+
Broad nose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Anteverted nares	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Large ears	…	−	…	…	…	+	…	+	…	−	+	−	…	…	−	+	…	+	+	−	+	+	…	+	…	+
Hypertelorism	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Downward-slanting palpebral fissures	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thick/everted lips	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Large mouth	+	−	…	+	+	…	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Frontal bossing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maxillary hypoplasia	…	+	…	+	+	+	+	…	…	…	+	+	+	+	+	+	…	+	…	+	+	+	+	…	…	…
High vaulted/narrow palate	…	…	…	+	…	−	+	…	…	…	+	…	…	+	…	…	…	…	+	…	…	…	+	+	…	+
Limbs:																										
Large, soft hands	+	+	…	…	+	+	+	+	+	+	+	+	…	+	…	…	…	+	+	+	+	+	+	+	+	…
Short, puffy, tapered fingers	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
Hyperextensible joints	−	+	+	+	+	…	…	…	+	…	…	+	+	…	…	+	+	…	+	+	+	+	…	+	−	+
Transverse hypothenar crease	−	…	…	…	…	−	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	+	…	+
Forearm fullness	…	+	…	…	…	…	…	…	+	+	+	…	…	…	…	…	…	…	…	…	…	…	…	+	…	+
Flat feet	−	…	…	…	+	+	…	…	+	+	…	…	+	+	…	…	…	+	+	…	+	+	…	+	+	+
Thorax:																										
Pectus carinatum/excavatum	…	+	…	…	…	…	…	−	…	−	…	−	+	−	−	…	…	−	+	…	…	…	−	…	…	…
Kyphosis/scoliosis	−	+	…	+	+	+	…	−	+	−	+	+	−	+	+	…	…	…	+	+	…	…	−	+	…	+
Cardiac:																										
Mitral regurgitation	−	+	−	…	…	…	…	…	…	−	−	−	…	−	…	−	+	+	…	…	−	…	−	…	+	…
Teeth:																										
Anodontia/oligodontia	−	−	−	…	+	+	+	−	−	−	+	+	+	−	−	+	…	+	+	+	…	…	+	…	+	−
Abnormal dental position	−	+	+	+	+	+	…	+	+	−	+	+	…	+	−	+	…	…	+	…	…	…	−	…	+	+
Radiology:																										
Drumstick terminal phalanges	−	+	…	+	+	+	+	+	…	+	+	+	…	…	+	+	−	+	+	+	+	+	−	+	+	+
Retarded bone age	…	…	…	…	+	+	…	+	…	+	+	…	…	…	…	−	…	…	…	…	+	+	+	…	+	+
Ligamenta flava calcification	−	…	…	…	…	−	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Narrow intervertebral spaces	−	…	…	…	…	…	…	…	…	+	…	…	…	…	…	…	…	…	…	…	…	…	…	+	…	…
Anterior vertebral body defect	…	…	…	…	…	…	…	…	…	+	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Thickened skull	…	…	…	…	…	…	…	…	…	+	…	…	…	…	…	…	…	…	…	+	…	…	…	…	…	…
Other features:																										
Hernia	…	…	+	…	…	…	+	…	…	…	+	…	…	+	…	+	…	…	…	…	+	…	+	…	+	…
Obesity	…	…	…	…	…	−	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	+
Autism	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	−	−	…	…	…	…	…	+	…
Expression in mother:	MM	NM	NM	NM	MM	NM	NM	MM	MM	NM	…	NM	MM	…	NM	NM	MM	MM	MM	NM	MM	…	…	MM	NM	NM

Note.—+ = Positive; − = negative; S = severe mental retardation, Mi = mild mental retardation; Mo = moderate mental retardation; MM = mild manifestation; NM = no manifestation; ellipses indicate that no data were available.

using the rabbit polyclonal anti-ERK2 K-23 antibody (Santa Cruz Biotechnology). Final detection was done by chemiluminescence by use of the Super Signal West Pico Stable Peroxide Solution (Pierce).

S6 Kinase Assay

The RSK2 protein was immunoprecipitated from protein extracts (1 mg total protein), by incubation for 2 h at 4°C with the rabbit polyclonal C-19 antibody (Santa Cruz Biotechnology), which was directed against the C-terminus of the human RSK2 protein. After incubation for 30 min with protein G sepharose, the immunoprecipitates were washed three times with NP40 lysis buffer and once with kinase assay buffer (20 mM 3-(N-Morpholino) propanesulfonic acid, with pH 7.2; 25 mM β-glycerol phosphate; 5 mM ethyleneglycoltetraacetic acid; 1 mM Na_3VO_4; and 1 mM dithiothreitol). The amounts of immunoprecipitated RSK2 protein were subsequently quantified on western blot, and quantities were normalized before performing an S6 kinase assay (S6 kinase assay kit; Upstate Biotechnology), as described elsewhere (Merienne et al. 1998). Five lymphoblastoid (from patients AG96, AZ21, CN14, DX6, and DX57) and four fibroblast (from patients CV59, DX34, DZ57, and MC9) cell lines were used as negative controls. Patients AG96, CN14, DX6, DZ57, and MC9 carry missense mutations (S227A, G75V, T231I, R383W, and I189K) that were shown to abolish or dramatically decrease the RSK2 kinase activity (Trivier et al. 1996; Merienne et al. 1998; Delaunoy et al. 2001). S227A, T231I, and R383W affect phosphorylation sites that are critical for RSK2 catalytic function, and G75V affects the N-terminal ATP binding site. I189K is likely to disrupt the secondary structure of the N-terminal kinase domain (Delaunoy et al. 2001). An intragenic deletion that encompasses exons 6 and 7 of the *RSK2* gene and results in a truncated nonfunctional protein was identified in patient AZ21. The remaining patients (DX57, CV59, and DX34) carry splice-site mutations (IVS6-1 G→A, IVS4+3 insT, and IVS14+2 T→C, respectively) (Trivier et al. 1996; Delaunoy et al. 2001). All of them result in truncated nonfunctional RSK2 proteins.

RT-PCR Analysis and Exon-Skipping Detection

RNA was extracted from cell lines by use of the RNA-Solv reagent (Omega Biotek). The first-strand cDNA was synthesized using both random hexamers and oligo-dT as primers. The primers used for PCR amplification were designed from the RSK2 cDNA sequences (Bjorbaek et al. 1995), with forward primers 5′-TGGCGCAGCTGGCGGA-3′, 5′-GATGTTCACAGAAGAAGAT-3′, 5′-ACCTCAGATGATGAAAGC-3′, 5′-GGAGGTGAATTGCTGGATAAAA-3′, and 5′-GCTATGATGCTGCTTGTGAT-3′ and reverse primers 5′-ATTATTCCCAGGCTATGTAG-3′, 5′-GCTTTCATCATCTGAGGT-3′, 5′-AGTTATAGTGAACAGGACAG-3′, 5′-GTATCATCAGGACCATTTGC-3′, and 5′-CAAATATCTCACTGAGGTCAC-3′.

PCRs were run for 35 cycles, and PCR products were resolved on 2% agarose gels. Electrophoretic variants were sequenced from both the forward and the reverse strands, after PCR products had been purified with the NucleoSpin Extract 2 in 1 kit (Macherey-Nagel).

Mutation Detection

Genomic DNA was extracted from patient cell lines for which exon skipping was observed. The exon or exons skipped, as well as the exons preceding and following, were PCR amplified using flanking intronic primers (Jacquot et al. 1998). The PCR products were sequenced after purification with the NucleoSpin Extract 2 in 1 kit (Macherey-Nagel). Exonic mutations were identified after direct sequencing of the RT-PCR products obtained as described above.

Promoter Sequence

The promoter sequence of the human *RSK2* gene was obtained after subcloning in a cosmid and direct sequencing of an *Eco*RI genomic fragment that contains $\geqslant$2,000 bp of sequence upstream from the translation start site.

Localization of the Transcription Start Site by RT-PCR

RNA from control lymphoblastoid cell lines was prepared as described above. RNA extraction was followed by 1 h DNase I treatment at 25°C. The first-strand cDNA was synthesized using reverse primers 5′-AGCAGCAGCAGCAGCGGC-3′ and 5′-TCCGTCCCGCCCGAGCC-3′. For PCR amplification, the primers used were as follows: forward primers 5′-GCGGAAGGAGGAGAGGAA-3′, 5′-CCGGTGCCCAGCGGAGGC-3′, 5′-GCGCGAGGCTCTCGCTCA-3′, 5′-CTGTTTCTTCTGGGAGCGG-3′, and 5′-AGAGGGAGGAGCGGAGCG-3′ and reverse primer 5′-AGAGGGCTCGACGCCGAC-3′. PCRs were run for 35 cycles. The PCR products were resolved on 2% agarose gels.

Primer Extension

The primers used were as follows: 5′-ACGCCGACGCCGACCGCCCGAAAGCCGCGC-3′ and 5′-GCTCGACGCCGACGCCGACCGCCCGAAAGC-3′. One hundred thousand counts per minute ^{32}P-labeled primer was hybridized to 30 μg total RNA, prepared as described above, from control lymphoblastoid cell lines. Reverse transcription was performed for 1 h at 42°C by use of the reverse transcriptase AMV (Boehringer Mannheim).

Table 2

Western Blot Results on Extracts from 26 Independent Patient Cell Lines

Patient	Cell Line[a]	RSK2 Protein[b]
AD57	F	−
CV59	F	−
DX43	L	+
DX55	F	+
DX86	L	+
DZ32	L	+
DZ36	L	+
DZ37	L	+
DZ75	L	−
DZ91	L	−
EZ14	L	+
EZ48	L	−
FP41	F	+
FP67	L	+
GM10	L	+
GM48	L	+
GM49	L	+
GM98	L	+
GW11	L	+
GW15	L	+
GW24	L	+
GW44	L	+
GW55	L	−
GW85	L	+
GW87	L	−
MH55	L	+

[a] F = fibroblast cell line; L = lymphoblastoid cell line.

[b] + = Present; − = absent.

The reaction products were subsequently resolved on 6% denaturing polyacrylamide gels and revealed by autoradiography.

Prediction of Transcription-Factor Binding Sites

For the search of consensus DNA elements involved in the regulation of gene expression, the following programs were used: MatInspector V2.2 (based on TRANSFAC 4.0) and Transcription Element Search (TESS).

Promoter-Region Mutational Analysis by SSCP

Genomic DNA was obtained from 66 independent patients with a clinical diagnosis of CLS and from controls. The promoter region of the *RSK2* gene was PCR amplified using four distinct sets of primers (forward primers 5′-CGCAGTGAACACAGGGTCGAGT-3′, 5′-ACCTCCCTGTTTTCCCCTCTTGA-3′, 5′-TACTTTGCCCACCTCCCT-3′, and 5′-GGGGGTGGGAAGAGGGAG-3′ and reverse primers 5′-TAAGGAAACCCAATGCCCCGAC-3′, 5′-CTCCTGGGGGCTCGGCTC-3′, 5′-CTCCGCTCCTCCCTCTTC-3′, and 5′-TCTCCTCCTTCCGCCGCC-3′). PCR products were analyzed by SSCP on PlusOne precast acrylamide gels (Pharmacia) with a Genephor electrophoresis device (Pharmacia), at two different running temperatures (5°C and 15°C). Electrophoretic variants were sequenced from both the forward and the reverse strands, after PCR products had been purified as described above. Presence or absence of mutations was tested by SSCP on control DNA from the respective populations.

Results

Western Blot Analysis and Identification of Seven Novel RSK2 *Mutations*

A series of 26 patients who exhibit features highly suggestive of CLS (table 1) and from whom lymphoblastoid or fibroblast cell lines were available were analyzed by western blot. For all these patients, previous SSCP analysis failed to detect any obvious abnormality in the coding region of the *RSK2* gene. However, an intragenic deletion was suspected in patient GW55, because of the failure to PCR amplify exon 18.

No RSK2 protein was detectable in 7 of the 26 patients analyzed (table 2). Three of the patients (DZ75, EZ48, and CV59) are shown in figure 1. The amount of ERK2, which was used as an internal control, was roughly the same in all the patients tested, as well as in positive and negative control subjects (for the same type of cell line), which excluded protein degradation in the negative samples. RNA was extracted from the seven cell lines, and RT-PCR products, covering the whole human RSK2 cDNA, were directly sequenced. This revealed the presence of transcripts internally deleted in four individuals (fig. 2). In patient DZ75, skipping of either exon 10 alone or both exons 9 and 10 was observed, whereas, in patients CV59, GW87, and GW55, skipping of only one exon (exons 4, 6, and 18, respectively) was evident. The exon organization of the RSK2 coding sequence predicts that all but one (in patient GW55) of these defects results in the alteration of the

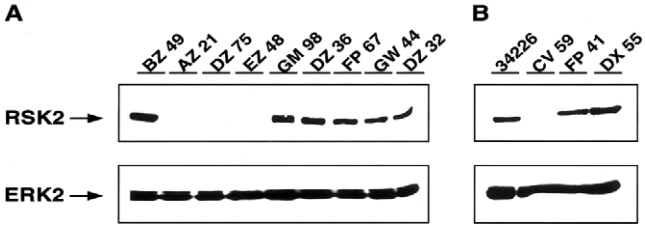

Figure 1 Western blot analysis of endogenous RSK2 (*top*) and ERK2 (*bottom*) proteins from lymphoblastoid (*A*) or fibroblast (*B*) cell lines, established from patients and controls. The origin of the cell line is indicated. Positive control cell lines shown are from patients BZ49 and 34226; negative control cell line shown is from patient AZ21. Three (patients DZ75, EZ48, and CV59) of the seven patients in whom the full-length RSK2 protein could not be detected are shown.

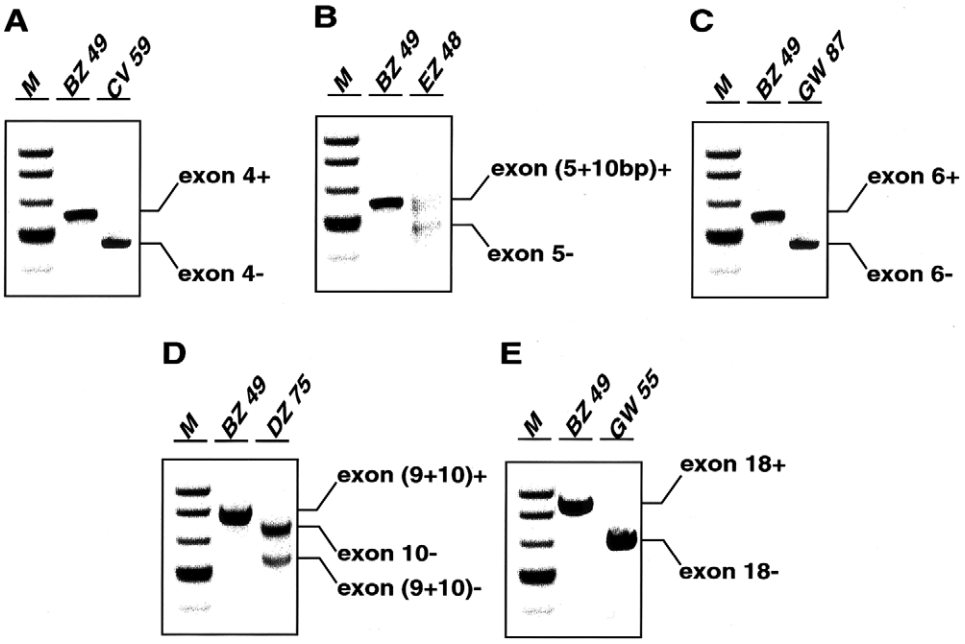

Figure 2 Abnormal transcripts identified by RT-PCR analysis in patients without detectable RSK2 protein. Plus signs (+) indicate the presence of the indicated exon(s), and minus signs (−) indicate the absence of the indicated exon(s). *A,* Absence of exon 4 in patient CV59. *B,* Skipping of exon 5 (*lower band*) and insertion of an additional 10 bp between exons 4 and 5 (*upper band*), in patient EZ48. *C,* Absence of exon 6 in patient GW87. *D,* Absence of either exon 10 (*upper band*) or both exons 9 and 10 (*lower band*), in patient DZ75. *E,* Absence of exon 18 in patient GW55.

reading frame, which leads to the introduction of a premature termination codon. Since the E1 antibody is directed against the carboxyl-terminus of RSK2, detection of truncated proteins is not expected, if the latter is present; this explains the absence of signal on western blot. Patient EZ48 showed two aberrant RT-PCR fragments, which were 10 bp larger and 81 bp smaller than expected (fig. 2). Direct sequencing of these fragments revealed that the larger fragment represents an alternatively spliced mRNA product in which a cryptic splice site, 10 bp upstream from the normal IVS4 acceptor splice site, has been used. The insertion of an additional 10 bp between exons 4 and 5 is predicted to cause a frameshift with premature termination in exon 5. The smaller fragment represents the skipping of exon 5. In both patients, EZ48 (for the smaller fragment) and GW 55, skipped exons (exons 5 and 18, respectively) are in frame. Absence of signal on western blot in those latter cases is probably due to instability of the resulting proteins.

Skipped exons, as well as preceding and following exons and respective surrounding intronic regions, were sequenced after PCR amplification of genomic DNA from the patients (fig. 3). This allowed us to identify four intronic mutations, including IVS6+3 A→G in patient GW87, IVS4+3 insT in patient CV59, and IVS10+5 G→A in patient DZ75; the fourth intronic base-pair change, IVS5-11 A→G, was observed in patient EZ48. In all these patients, no other sequence abnormality was observed, indicating that these changes are indeed responsible for the splicing defects observed. Sequencing of RT-PCR products from patient GW55 revealed absence of exon 18 in the *RSK2* mRNA. We were unable, repeatedly, to amplify exon 18 with flanking primers from genomic DNA from this patient, suggesting that an intragenic deletion encompasses exon 18 and at least part of intronic sequences surrounding this exon. This was confirmed by subsequent Southern blot analysis (not shown). Direct sequencing of the RT-PCR products from patients AD57 and DZ91 revealed the presence of mutations in the coding region of the *RSK2* gene. A single-nucleotide insertion in exon 4 (262 insA), resulting in a frameshift, was found in patient AD57. Patient DZ91 carried a missense mutation in exon 21 (1964 T→C), resulting in the substitution of a leucine by a proline residue (L655P).

All the patients in whom the RSK2 protein was present at normal levels were further investigated using the S6 kinase assay (fig. 4). Negative controls included patients with CLS with already characterized *RSK2* mutations, which resulted either in a dramatic decrease of the RSK2 kinase activity—since they affect the catalytic domains, the ATP binding sites, or the regulatory domains of the protein—or in truncated nonfunctional RSK2 proteins (Delaunoy et al. 2001).

Of the 19 patients tested, 4 (patients DZ36, DZ37, GM98, and MH55) presented reduced but not abolished RSK2 kinase activity (fig. 4). However, after RNA ex-

traction from the lymphoblastoid cell lines that were established from these patients and direct sequencing of RT-PCR products, we could not find any abnormality in the coding region of the *RSK2* gene.

In some patients, a higher RSK2 kinase activity was found, when compared to positive controls (fig. 4). In all these patients, levels of RSK2 protein as determined by western blot analysis were very similar to those of normal control individuals. In addition, semiquantitative RT-PCR analysis failed to reveal higher levels of *RSK2* mRNA (not shown) in these patients. Together, our results exclude the possibility of duplication of the *RSK2* gene in any of these patients, and direct sequencing of the entire *RSK2* cDNA in two of the patients (GW11 and GW85) failed to identify any sequence abnormality.

Study of the Promoter Region of the Human RSK2 *Gene*

Our results confirm that, for the *RSK2* gene, as expected from results for other genes, some mutations (mostly intronic ones) are missed by SSCP analysis. However, this cannot fully explain our incapacity to identify mutations in such a large number (66%) of patients in whom CLS features were diagnosed. This prompted us to explore the possibility of mutations in the promoter region of the *RSK2* gene. Mutations that affect transcription of the *RSK2* gene in lymphoblasts or fibroblasts are expected to be detectable by western blot analysis. However, mutations that would affect RSK2 protein expression in other cell types would be missed by such analysis.

Since the nucleotide sequence of the promoter region was not yet known, we have determined the sequence of a region of 2,000 bp upstream from the translation start site (fig. 5). To identify the transcription initiation site, we performed RT-PCR experiments on RNA from a human lymphoblastoid cell line, in which the gene is well expressed. Our results indicated that the start site of transcription is located between positions −452 and −297, with respect to the first nucleotide of the translation initiation codon, which has been denoted as "1." Indeed, using a primer starting at position −297, we were able to amplify a fragment of the expected size, whereas, using a primer starting at position −452, we obtained no amplification product (not shown). Primer-extension experiments allowed us to precisely identify the transcription initiation site; it is located at the first guanosine of the sequence 5′-GCGGTC-3′, at position −361 (fig. 5). This is in agreement with the results obtained by RT-PCR. The 5′-flanking region of the *RSK2* gene is particularly (78%) GC rich. In addition, no identifiable CAAT-box or TATA-box motifs were found immediately upstream from the transcription initiation site. These features are common to the regulatory sequences of housekeeping genes. This is in agreement with the observation of ubiquitous expression of the *RSK2* gene (A.H., unpublished results).

To identify putative *cis*-acting transcription-factor binding sites, we searched sequences spanning positions

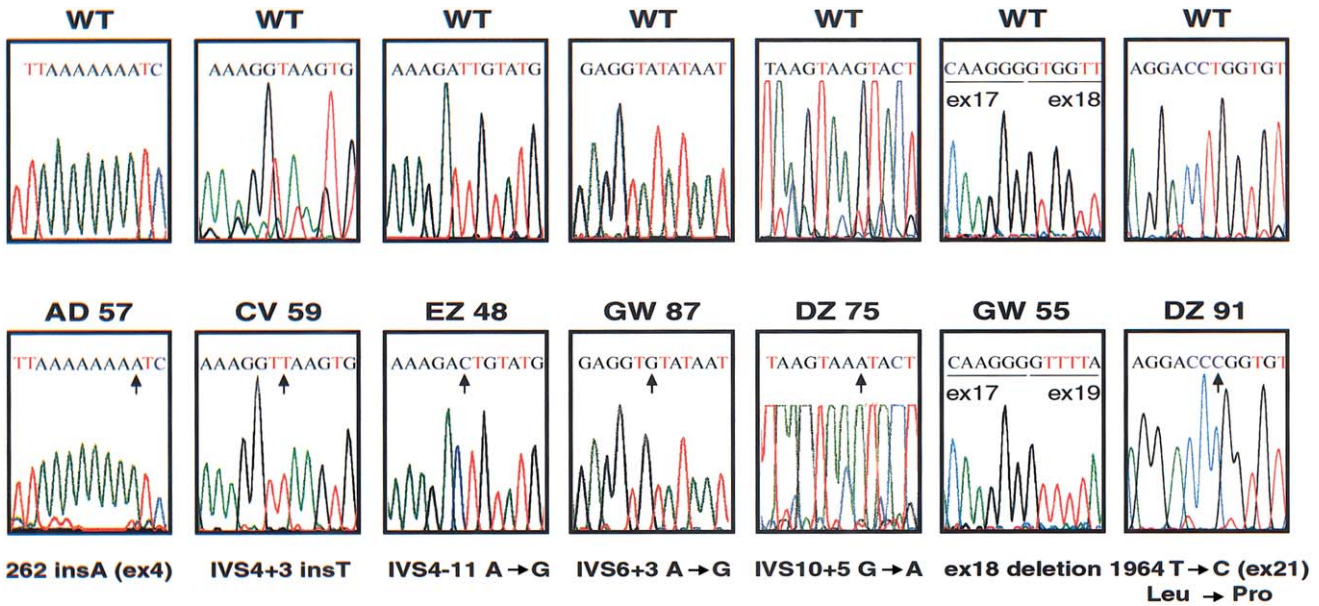

Figure 3 *RSK2* sequence variants in seven patients with no detectable RSK2 protein. Wild-type (*top panels*) and mutant (*bottom panels*) sequences are shown. Arrows indicate the site of mutation. The mutations identified are indicated below the lower panels. Forward sequences are given for all patients except patient EZ48.

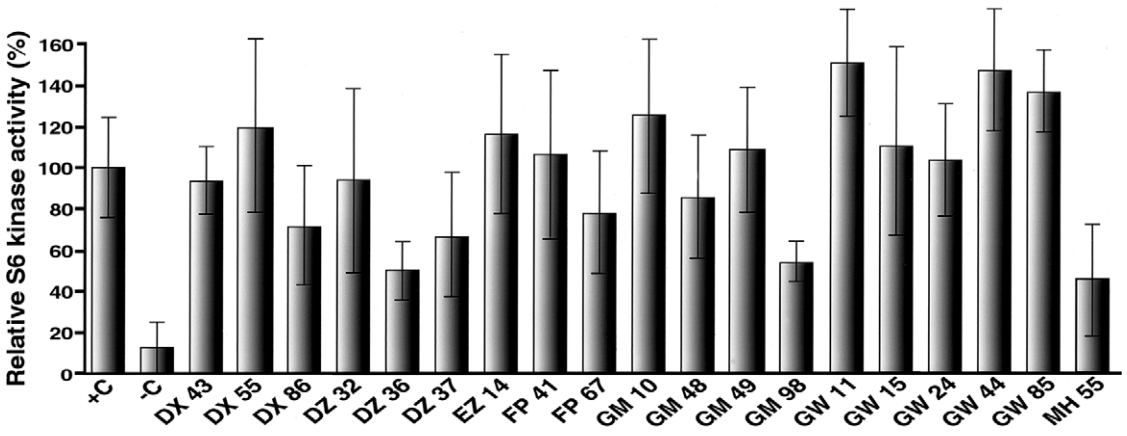

Figure 4 RSK2 enzymatic activity in 19 patients. RSK2 was immunoprecipitated from equal amounts of total proteins derived from lymphoblastoid or fibroblast cell lines from patients who presented normal levels of RSK2 protein on western blot. The values obtained for positive and negative controls (+C and −C, respectively) in lymphoblastoid and fibroblast cell lines were comparable. Data are represented as the percentage of the mean specific RSK2 activity immunoprecipitated from four wild-type lymphoblastoid and two wild-type fibroblast cell lines (+C; wild-type relative–specific activity 100% ± 26%). Negative controls (see "Patients and Methods") included five lymphoblastoid and four fibroblast cell lines (−C; mean value 13.8% ± 13.3%). Experiments were repeated at least three times with independently prepared cell extracts. The limit lines associated with each bar represent the SD from the mean value for all experiments.

−1800 to 1, with both the MatInspector program and the TESS program. Several Sp-1 binding sites were predicted by both programs, in agreement with the GC-rich nature of the promoter region. Additional putative consensus motifs identified include a c-Ets-1 binding site, a Pu-box normally bound by the Ets-family macrophage-specific factor PU.1, as well as binding sites for factors such as AP-4, AP-2, E2A, the glucocorticoid-receptor/prolactin-receptor dimmer, and the estrogen receptor. However, further functional studies are necessary to determine whether these motifs have a physiological relevance.

Search for Mutations in the Promoter Region of the Human RSK2 *Gene by SSCP Analysis*

Four distinct regions located in the promoter of the *RSK2* gene were amplified by PCR from genomic DNA extracted from the cell lines in which no *RSK2* mutation has been found. In addition, blood-extracted genomic DNA from 47 independent patients diagnosed with CLS for whom previous SSCP analysis failed to identify a mutation in the coding region of the *RSK2* gene was also analyzed. Promoter regions that were screened contain putative transcription-factor binding sites and/or the transcription start site. SSCP analysis of the resulting fragments revealed two rare polymorphisms: one at position −1140 (C instead of T) and one at position −320 (C instead of G) immediately upstream from a predicted Sp-1 binding site. The first polymorphism was observed in patient DZ75 and on 1 of 88 normal control X chromosomes tested; the second polymorphism was found only in patient DZ32 but not in 134 normal control X chromosomes tested. Semiquantitative RT-PCR experiments failed to show reduced RSK2 mRNA expression in the lymphoblastoid cell line derived from this patient (not shown). In addition, western blot analysis and S6-kinase-assay tests revealed normal levels of RSK2 protein and normal RSK2 phosphotransferase activity (figs. 1 and 4). No other type of cell line is currently available from this patient. Together, these results suggest that this latter nucleotide change may be a rare polymorphism and not a disease-causing mutation.

Discussion

We have shown previously that CLS is caused by loss-of-function mutations in the *RSK2* gene. On the basis of current knowledge on the nature of *RSK2* mutations, it can be estimated that western blot analysis allows detection of up to 70% of the mutated RSK2 proteins (Delaunoy et al. 2001). Most of the remaining *RSK2* mutations are missense changes that have been shown to result in partial or complete loss of RSK2 phosphotransferase activity when tested in an in vitro S6 kinase assay (Trivier et al. 1996; Merienne et al. 1998). In the present article, we have further investigated the efficiency of western blot analysis and kinase assays as tools in the diagnosis of CLS, as well as the proportion of patients with RSK2 deficiency that have not been detected by SSCP analysis. Another important issue that we wanted to address is the possible genetic heterogeneity in CLS.

Western blot analysis revealed absence of the full-length RSK2 protein in approximately one-fourth (27%) of the patients included in this study. More than half of the mutations subsequently identified are found in intronic regions and do not affect the consensus GT or

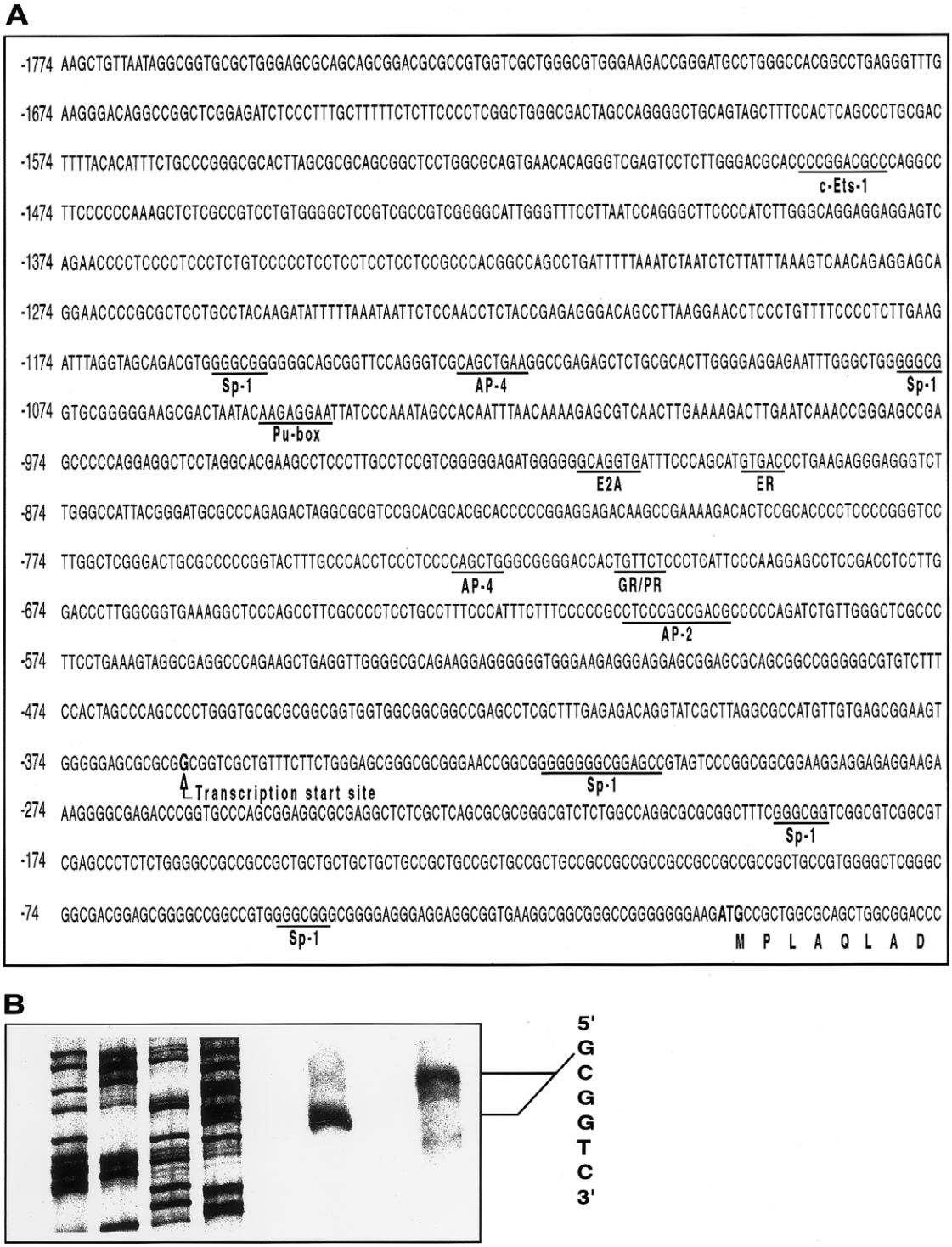

Figure 5 Characterization of the 5′ end of human *RSK2*. *A*, Representation of the 5′ sequence from *RSK2*. The transcriptional start site is indicated in boldface, at position −361; the translational start site (ATG with A at position 1) and coding sequence for human RSK2 are denoted in boldface. Underlined sequences correspond to putative *cis*-acting transcription-factor binding sites, as predicted by the MatInspector and TESS programs. *B*, Determination of the transcriptional start site. Two distinct 30-mers were elongated by primer extension and were resolved along with a sequencing ladder. The transcriptional start site was determined relative to the length of the resulting fragments.

AG splice sites. Thus, even if they had been identified by SSCP analysis, further investigations—such as RT-PCR, which cannot be performed easily in a routine diagnostic laboratory—would have been necessary to determine whether they affect normal splicing. Interestingly, two of these mutations are quite unusual:

1. To our knowledge, an A→G transition at position 3 of a 5′ donor splice site (as had been observed in patient GW87) has been documented in only a small number of human disorders (Ohno et al. 1999; Park et al. 2000). This mutation, which results in skipping of the nearby exon, is puzzling because both A and G nucleotides are commonly observed at position 3 of native splice-donor sites in human (Ohno et al. 1999).
2. In patient EZ48, we observed an A→G transition at position −11 upstream from exon 5, creating a cryptic acceptor splice site which is preferentially used. Indeed, we believe that the low visible amount of the resulting transcript on the gel is due to degradation by nonsense-mediated mRNA decay. In some molecules, the mutation leads to skipping of exon 5.

Both of these mutations are currently being further investigated to determine the mechanisms that lead to these intriguing aberrant-splicing events. Patients CV59 and DZ75 bear mutations that affect the donor splice sites downstream from exons 4 and 10 (i.e., IVS4+3 insT and IVS10+5 G→A), respectively, thereby leading, in both cases, to complete skipping of the nearby exon(s).

The high proportion of four splicing aberrations in a total of seven mutations that have been identified in this study is noteworthy. Abnormal mRNA splicing does indeed represent a relatively common mechanism in the pathogenesis of CLS. Altogether, splice-site mutations account for 24% of all mutations so far identified in patients with CLS, which is much higher than the 10% that can be calculated when taking into account all the gene mutations that are listed in the Human Gene Mutation Database (Krawczak and Cooper 1997).

Interestingly, we were able to identify two patients who carried mutations in the coding region of the *RSK2* gene that were not detected by SSCP analysis. Patient AD57 carried a frameshift mutation in exon 4 (262 insA), and patient DZ91 carried a missense mutation (1964 T→C) in exon 21 that lead to the substitution of a leucine residue by a proline residue at codon 655. Since the RSK2 protein is not detectable by western blot analysis in the latter case, we assumed that the L655P missense mutation affects the structure of the RSK2 protein, thereby leading to its instability. This is the first instance of a missense mutation in the *RSK2* gene that results in lack of the RSK2 protein. However, missense mutations that lead to unstable proteins have already been described in other genetic disorders (Laporte et al. 2000).

Finally, patient GW55 carries a genomic deletion that encompasses exon 18 and at least part of the surrounding introns. Only two large deletions had previously been identified in the *RSK2* gene (Delaunoy et al. 2001), thus making this the third such deletion.

Cell lines from 19 patients who showed normal levels of RSK2 protein by western blot analysis were subsequently assayed for RSK2 phosphotransferase activity. None of the patients presented a dramatically reduced RSK2 activity. Mutations that affect RSK2 catalytic domains, regulation sites, or the ERK binding site have been shown to be constantly associated with either complete loss or up to 80% loss of enzymatic activity (Trivier et al. 1996; Merienne et al. 1998, 1999; Manouvrier et al. 1999). These results have indicated that the S6 kinase assay is capable of detecting with confidence more than 80% of reduction of kinase activity. Thus, it is unlikely that patients studied in the present article carry such severe mutations. Some patients (DZ36, DZ37, GM98, and MH55) showed diminished, although not dramatically reduced, kinase activity. Importantly, cDNA sequencing did not identify any mutations in the *RSK2* gene, and western blot analysis showed normal levels of protein expression in these patients. In addition, no nucleotide change was found in the promoter region. We speculate that these patients may carry mutations in genes that are distinct from *RSK2* and encode proteins that, for instance, act upstream from RSK2 in the signaling pathway or are involved in the regulation of the RSK2 enzymatic activity. However, as yet, no mutations in such known genes have been found to be associated with a CLS-like phenotype. Fibroblast cell lines from these patients will be available in the near future, and their availability will allow the confirmation of the results in a different cell type and the further investigation of the MAPK-RSK2 pathway.

Finally, duplication of the *RSK2* gene was excluded in some patients with slightly increased levels of RSK2 kinase activity. Direct cDNA sequencing excluded the presence of mutations in two of these patients (GW11 and GW85).

Our data suggest that a mutation that results in a $\leqslant$50% decrease of kinase activity would be difficult to detect by use of the current protocol. Growth of Epstein-Barr virus–transformed lymphoblastoid cells is highly variable from one cell line to another and even from one experiment to another. In addition, the MAPK pathway and RSKs have been implicated in cell proliferation and cell-cycle control. This may explain the variations observed in the levels of kinase activity (for equal amounts of RSK2 protein) (fig. 4). Experiments with nontransformed (i.e., primary) fibroblast cell lines (which can be stimulated with growth factors

such as EGF [epidermal growth factor]) and some modifications of the protocol are ongoing, to try to improve this assay. No consistent relationship has been observed between specific mutations and the severity of the disease (Jacquot et al. 1998). Nevertheless, we have observed that there is a tendency for some patients who carry missense mutations that leave residual enzymatic activity to be less severely affected. The less severely affected male patient in the cohort with CLS that we studied expressed a mutant protein that retains 20% of residual kinase activity. This patient did not exhibit any of the facial, digital, and skeletal features of CLS, and his cognitive function was only mildly impaired (Merienne et al. 1999). Thus, together, our current data suggest that only very mild cases, which may not even currently be recognized as CLS on the basis of clinical examination, would probably be missed by the S6 kinase assay.

Indeed, our results confirm that western blot analysis of RSK2 is a very efficient, reliable, and rapid assay and allows confirmation of the diagnosis in most patients with CLS. Moreover, as indicated by the present data, even patients who carry some missense mutations may be detected by immunoblot analysis. Thus, this assay may be a very useful prescreening test for CLS, in medical practice. We have previously shown that the RSK2 protein can be detected using lymphocyte-protein extracts that were prepared directly from fresh blood samples (i.e., those extracted within 24 h) (Merienne et al. 1998). In addition, western blot analysis can be used for prenatal diagnosis, since the RSK2 protein is readily detectable in cultured amniocytes (Jacquot et al. 2002). Use of the kinase assay as a routine test is more questionable. It would certainly be the diagnostic method of choice, since it potentially detects all classes of mutations; however, it can be used only on either cultured fibroblasts or a lymphoblastoid cell line, and, given our previous (Merienne et al. 1998) and present results, it would probably detect with confidence only dramatic decreases of enzymatic activity. Therefore, we suggest that this test should be performed only for patients with typical CLS features in whom the western blot analysis reveals a normal level of RSK2 protein.

We have cloned and sequenced the human RSK2 promoter region. It has been shown that the RSK2 protein is expressed in a wide variety of tissues in human and mouse. However, it is expressed at much higher levels in skeletal muscle, heart, and pancreas (A.H., unpublished results), suggesting that the *RSK2* gene may be differentially regulated in a tissue-specific manner. Consistent with the broad tissue expression, the RSK2 proximal promoter region has the typical features of a housekeeping gene. It lacks canonical TATA and CAAT boxes but exhibits several GC boxes, which are potential binding sites for ubiquitous transcription factors such as NFκB and Sp1. Interestingly, also putative Ets binding sites have been identified. Further studies will be required to determine if these motifs have a functional significance. Analysis of the RSK2 putative promoter region, in 66 patients, revealed only two very rare polymorphisms. This result makes it unlikely that mutations in the promoter region could account for a high number of patients.

The hypothesis of additional exons not yet discovered, resulting from alternative splicing, which would contain the missing mutations is also very unlikely. BLAST screening of human and mouse EST databases does not reveal any RSK2 ESTs with additional still-unknown sequences. In addition, western and northern blot analysis in various mouse tissues (S. Jacquot and A.H., unpublished results) do not provide any suggestion of alternative splicing.

No *RSK2* mutation has been detected in 66% (165/250) of the patients who were investigated by SSCP analysis (Delaunoy et al. 2001). Even if mutations have been missed by SSCP in 27% of these patients, as suggested by the present article, 50% of patients who have no *RSK2* defect remain. All of these patients were referred by various geneticists for mutation screening because the patients expressed clinical features that were highly suggestive of CLS. We were able to obtain detailed clinical records for only a minority of these patients. Since it is not always easy to distinguish CLS from other syndromes involving mental retardation—especially in milder cases and cases affecting young children—it is likely that some of these individuals were misdiagnosed. In particular, similarities in the clinical presentation of CLS and X-linked α-thalassemia mental retardation (ATR-X) have been repeatedly noted. However, given the high proportion of patients without an *RSK2* mutation, the possibility of locus heterogeneity should also be taken into consideration. Interestingly, a patient with a CLS-like phenotype who carried an interstitial deletion on chromosome 10 has recently been reported (McCandless et al. 2000). This patient was of short stature, had a coarse facial appearance similar to that of patients with CLS, and had hand findings that were characteristic of CLS. In our previous linkage studies, we used some of the rare known large pedigrees with CLS, and, at that time, there was no suggestion of genetic heterogeneity (Hanauer et al. 1988; Biancalana et al. 1992, 1994). Subsequent investigation revealed that, in fact, most families with CLS are sporadic cases, as are 80% of the patients referred to our laboratory for mutation screening. In addition, in most of the pedigrees with two (or, in very rare cases, more) affected family members, often only one was available for analysis. Thus, genetic heterogeneity could not be further investigated by linkage studies.

Although very well documented in a few patients

only, clinical data have been available for the 26 unrelated patients included in this study. All present with the major hallmarks of CLS (table 1). One patient (GW85; age 28 years) exhibited autistic behavior, a feature that has never been documented in patients with CLS. In addition, he apparently did not exhibit hypotonia. However, he presented with typical face and hand anomalies. Prader-Willi syndrome was excluded in this patient. Review of the available medical records revealed no obvious phenotypic differences between individuals who did or did not have an *RSK2* mutation. None of the patients who did not have an *RSK2* mutation exhibited α-thalassemia and/or urogenital abnormalities, excluding at least the classical form of ATR-X. In addition, examination of photographs (available for approximately two-thirds of patients) confirmed, in most of the these patients, the presence of characteristic large, soft hands and downward-slanting fissures (table 1), two features that are not found in ATR-X. Finally, mothers of eight patients without an *RSK2* mutation showed mild manifestations (table 1), in contrast to ATR-X carriers, who usually show no manifestations. ATR-X has been further excluded, on the basis of mutation screening, in three patients (DZ36, GM49, and GW85).

All patients except DX43, DX55, DZ36, and DZ37 have sporadic CLS. The affected brother of patient DX55 died a few years ago, and no blood sample from the unaffected brother of patient DZ36 could be obtained for analysis. Patient DX43 has one affected brother, and patient DZ37 has one unaffected brother and one unaffected sister. In all of the latter patients, segregation analysis of *CLS*-flanking markers is compatible with linkage to the *CLS* locus.

It cannot be excluded that some mutations affect particular RSK2 functions without affecting its enzymatic activity as assessed in our in vitro test. However, *RSK2* cDNA from six patients who showed a typical CLS phenotype and in whom the RSK2 protein was present was completely sequenced and did not show any abnormality. Thus, our data suggest the existence, in another gene (or genes), of defects that result in a CLS-like phenotype. Further studies need to be undertaken before a definitive conclusion about genetic heterogeneity can be drawn. Global assessment of gene-expression levels by use of high-density microarrays that represent all human genes may be used to identify over- or underexpressed candidate genes, for more-detailed studies in these patients.

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Electronic-Database Information

The accession number and URLs for data in this article are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/BLAST/ (for human and mouse databases)
Coffin Lowry Syndrome Mutations Data Base, http://www-ulpmed.u-strasbg.fr/chimbio/diag/coffin/index.html
MatInspector V2.2: Input Form, http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CLS [MIM #303600])
Welcome to TESS: Transcription Element Search System, http://www.cbil.upenn.edu/tess/

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