LETTER TO JMG

Molecular analysis of the *CBP* gene in 60 patients with Rubinstein-Taybi syndrome

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Rubinstein-Taybi syndrome (RTS, MIM 180849) occurs in 1/125 000 births and is characterised by growth retardation and psychomotor developmental delay, broad and duplicated distal phalanges of the thumbs and halluces, typical facial dysmorphism, and an increased risk of neoplasia.[1] RTS has been shown to be associated with chromosomal rearrangements in cytogenetic band 16p13.3,[2–4] all involving the CREB binding protein gene, officially named *CREBBP* by the HUGO Nomenclature Committee, but generally referred to by its shorter acronym *CBP*.[5] *CBP* is a transcriptional coactivator involved in different signal transduction pathways, thereby regulating the expression of many genes and playing an important role in the regulation of cell growth, cellular differentiation, and tumour suppression.[6 7] To date, all studies concerning *CBP* in RTS have used FISH analysis with cosmids from the *CBP* region or the search for mutations at the molecular level using the protein truncation test.[8 9] Taken together, these studies showed that translocations and inversions form the minority of *CBP* mutations in RTS, microdeletions account for only 10% of RTS cases, and PTT studies showed 10% truncating mutations. The structure of the *CBP* gene was recently described.[8] *CBP* spans about 150 kb with 31 exons and its cDNA is 9 kb in length.

We report here the use of different molecular techniques to analyse the *CBP* gene in a cohort of 60 RTS patients. These include cDNA probes to search for gross rearrangements by Southern blot analysis and to identify *CBP* mRNA of abnormal sizes on northern blots, intragenic microsatellite markers to look for intragenic deletions, as well as a complete series of primers to PCR amplify each of the 31 exons of the gene for mutation searching by direct sequencing. We have analysed 60 patients using these various techniques and identified 27 mutations.

METHODS

Sixty-three patients affected with RTS were selected from France, Belgium, and Switzerland. The cohort consisted of 36 female and 27 male patients. There were 58 white patients and five Arab patients. Careful examination of the patients' phenotypes indicated that all expressed the RTS phenotype

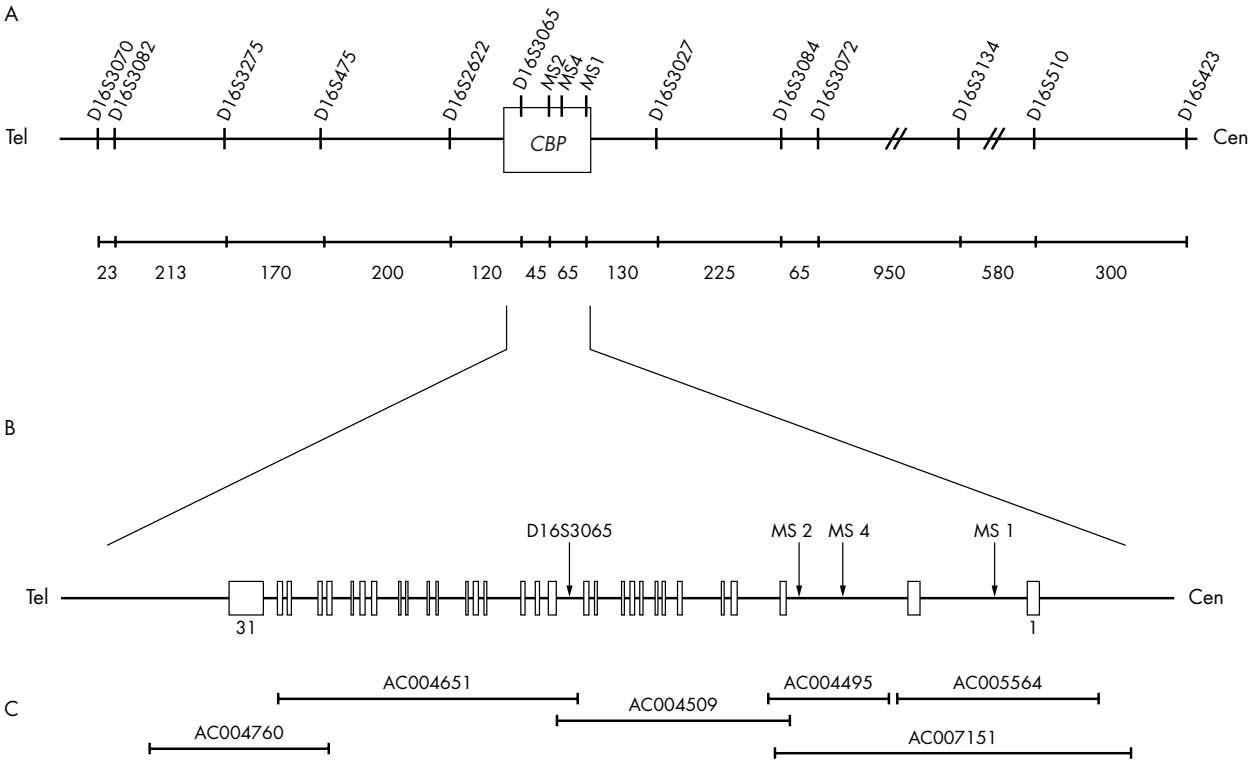

Figure 1 Microsatellite map of the cytogenetic band 16p13.3 region. (A) Position of microsatellite markers. Distances between microsatellite markers are given in kb. (B) Localisation of the intragenic microsatellite markers D16S3065, MS1, MS2, and MS4. (C) The BAC/cosmid contig of the *CBP* region.

Table 1 cDNA probes used to study the *CBP* gene in RTS patients

Probe	Position	Exons hybridised	Fragment size (bp)	Sequence 5′→3′	PCR conditions $MgCl_2$ - Annealing T
P1	–796, –777 +117, +137	1, 2	933	GAGGAATCAACAGCCGCCAT GGTATCAGCTCATCAGGAAGA	2.5 mmol/l - 60°C
P2	+33, +52 +1008, +1027	2-4	995	CCCCAAAAGAGCCAAACTCA CTGTTGCAATTGCTTGTGTG	2 mmol/l - 55°C
P3	+949, +968 +1914, +1933	4-9	985	ACTTCAGTCACCAACGTGCC CAGACTCGTACATGTCCCCT	1.5 mmol/l - 60°C
P4	+1847, +1866 +2967, +2985	9-15	1139	CACCTGATCCCGCAGCTCTA CACAGGTACGTCAGGTCCT	2 mmol/l - 60°C
P5	+2741, +2759 +3916, +3937	14-22	1197	AGAGCACCCCTACAGTCCA AGCAGTTGTCGCACACAAAACC	2 mmol/l - 60°C
P6	+3756, +3775 +4948, +4967	21-29	1212	GGGTGACGACCCTTCACAGC AGGGGGTCGGGGTCGACGAT	2 mmol/l - 60°C
P7	+4836, +4855 +5873, +5893	29-31	1058	CAACGTGTCCAATGACCTGT CTCGATCTGCCGAGCCGCTT	1.5 mmol/l - 55°C
P8	+5742, +5761 +7005, +7024	31	1283	CGTGAGCATGTCACCAGCTG TACTAAGGGACGTGGCGATC	2.5 mmol/l - 60°C
P9	+6903, +6922 +8214, +8235	31	1333	GATGAAGCAGCAGATTGGGT CCTTTTTGTCTGTTGCACACAG	1.5 mmol/l - 60°C

Positions of the primers are given relative to the start codon. The first primer of each set has a forward orientation, the second reverse.

including broad thumbs or halluces, typical facial dysmorphism, and mental retardation. Clinical data including a questionnaire, photographs, *x* rays, and medical records were collected for all patients when available. Phenotype details were obtained for 57 patients. Clinical assessment was made before knowledge of the molecular status. Blood samples were collected after obtaining fully informed consent from patients and their family. This study was authorised by the Comité Consultatif pour la Protection des Personnes dans des Recherches Biomédicales (CCPPRB) of Bordeaux.

RESULTS

Deletions of cytogenetic band 16p13.3 account for about 10% of RTS patients. Although deletions in RTS patients are routinely looked for by FISH analysis,[8 10] they can also be identified, and their extent characterised easily, by microsatellite marker genotyping. Several microsatellites have been described in the cytogenetic band 16p13.3. Their relative order, however, was discordant between the Genethon and Marshfield maps.[11] We have now taken advantage of the available contig mapping and large scale sequence data (htgs: high throughput genome sequence) to establish a precise physical map of the region. Marker order and physical distances (in kb) are shown in fig 1A.

BLAST alignment of D16S3065 with BACs AC004651 and AC004509 allowed us to localise it in intron 13 of the *CBP* gene at position 1183-1526 bp (relative to the first base of intron 13). Analysis of the genomic sequence available throughout the cosmid contig covering the *CBP* gene (GenBank accession No AC005564, AC007151, AC004495, AC004509, AC004651, AC004760) allowed us to identify three novel polymorphic repeats: MS1, MS2, and MS4 (fig 1B). MS1 was characterised by a $(GT)_n$ $(GA)_n$ repeat at position 8548-8687 of intron 1 (relative to the first base of intron 1). Six different alleles were identified by analysing 100 independent chromosomes (heterozygosity = 0.655). MS2 is a $(CA)_n$ N_{20} $(CA)_n$ at position 35997-36165 of intron 2 (relative to the first base of intron 2), characterised by eight alleles (heterozygosity = 0.795). MS4 is a $(GT)_n$ repeat at position 26238-26371 of intron 2 (relative to the first base of intron 2), characterised by seven alleles of which two represent 66% and 28%, respectively, of alleles (heterozygosity = 0.485). These microsatellites have been submitted to dbSTS with the Accession numbers G72365, G72366, and G72367 for MS1, MS2, and MS4, respectively.

Four microsatellite markers are therefore present in the first half of the *CBP* gene (MS1 - MS4 - MS2 - D16S3065). Sequence analysis of the 3′ half of the gene did not show any other polymorphic repeat.

The microsatellite map thus constructed was used to perform deletion analysis in four RTS patients for whom parental DNA was available. Patient 59 has a deletion encompassing markers D16S3065, D16S3084, D16S3072, MS1, MS2, and MS4, whereas markers D16S475, D16S2622, D16S3070, D16S3134, D16S510, and D16S423 were not deleted, and markers D16S3027 and D16S3082 were not informative. The deletion, which occurred on the paternal chromosome, was framed by markers D16S3134 on the proximal side and D16S2622 on the distal side and spanned at least 560 kb. In patient 9, D16S3275, D16S3065, and MS4 were not deleted. Marker MS1 was found to be deleted. MS2 and D16S3027 were not informative. The data indicated that the N-terminal region of the *CBP* gene was deleted in this patient and the deletion had occurred on the maternal chromosome. No deletion was found in patients 38 and 49.

Finally, patient 34 was known to be deleted for the RT100 probe, covering *CBP* exons 17 to 31,[8] by FISH analysis, suggesting a deletion of at least the 3′ end of the *CBP* gene. To refine the extent of the deletion, intragenic microsatellite marker analysis was performed. This patient was heterozygous for all intragenic markers, suggesting that the deletion was restricted to the 3′ end of the *CBP* gene.

Based on the published *CBP* mRNA sequence (9068 bp) (GenBank Accession No U85962, GI:4321115), nine pairs of primers were derived to amplify overlapping cDNA fragments of approximately 1 kb in length by RT-PCR (table 1). RT-PCR was performed on mRNA isolated from a control cell line and 18 RTS patients for whom a lymphoblastoid cell line was available. Specificity of the RT-PCR products was ascertained by sequence analysis. No abnormal fragment could be identified on RT-PCR products of these RTS patients, except for patient 7. Primers corresponding to probe P3 (table 1) showed

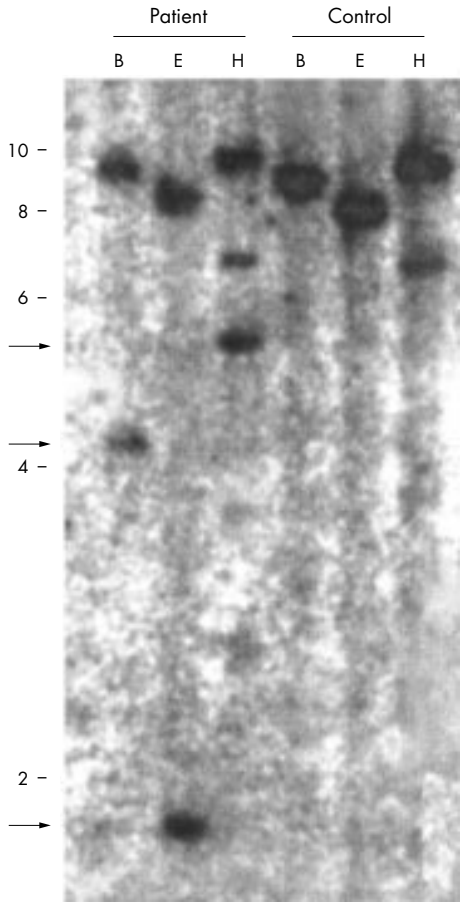

Figure 2 Southern blot analysis of RTS patient. DNA from patient 3 (left lanes) and from unaffected control (right lanes) was digested with *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). The Southern blot was hybridised with the P2 probe. Arrows indicate the additional fragments.

the presence of an abnormal fragment of 350 bp in addition to the normal fragment (985 bp) in the cDNA of this patient. The smaller fragment was electroeluted and sequenced. Sequence analysis showed a deletion of exons 4 and 5.

Looking for truncated mRNA by northern blot analysis on the same 18 patients, patient 1 was found to express a full length mRNA and a truncated form about 5.4 kb in length seen with probes P1, P2, and P5 but not with probes P6 or P9, suggesting a deletion of the 3' end of the gene (data not shown). However, Southern blot analysis of this patient did not show any abnormal restriction fragment with probes P2, 3, 4, 5, and 6. It is therefore possible that the truncated RNA is caused by an intronic mutation that remains undetected so far.

The cDNA probes were used to hybridise Southern blots carrying genomic DNA from a first series of 26 patients digested with three restriction enzymes (*Eco*RI, *Bam*HI, and *Hin*dIII). DNA from an unaffected control produced band patterns consistent with the *CBP* gene restriction map, including an 8.2 kb *Eco*RI fragment, an 8.8 kb *Bam*HI fragment, and 9.9 kb and 6.5 kb *Hin*dIII fragments. Patient 3 displayed an additional fragment with probe P2 at 1.8 kb, 4.2 kb, and 5.2 kb in *Eco*RI, *Bam*HI, and *Hin*dIII digests, respectively (fig 2). None of the other cDNA probes showed an abnormal band pattern. These data suggest the presence of a small rearrangement in intron 2 (39517 bp) of the *CBP* gene. Further characterisation of this rearrangement is under investigation by FISH analysis.

The *CBP* gene is composed of 31 exons scattered over a 150 kb genomic region.[8] Primers were derived from the *CBP* sequence (GenBank accession No AC005564, AC007151, AC004495, AC004509, AC004651, AC004760), to amplify each of the 31 exons separately, including the intron-exon boundaries (table 2). Exon 1 (890 bp) was difficult to amplify, probably because of its high G-C content. So far, only the amplification of the coding part of this exon could be achieved (−22 to +178 bp; the initiating ATG codon is at position 1). Similarly, exon 31 also proved difficult to amplify and the first 707 nucleotides of it has not been able to be amplified so far.

A mutation search was performed in a series of 60 patients by direct sequencing of all 31 exons. Twenty-two mutations were found, including four missense, 13 nonsense, and five splice site mutations (table 3). The missense mutations described in table 3 are discussed further. In patients 8 and 39, single nucleotide deletions in exons 14 and 30, respectively, produced a shift in the reading frame, leading to a stop codon 54 amino acids further on in exon 15 (patient 8) and 94 amino acids further on in exon 31 (patient 39). Single base pair insertions were found in three patients leading to a frameshift and a premature stop codon at the insertion level (patients 33 and 68) and 43 amino acids further on in patient 51. Patient 52 presented a single nucleotide deletion (A) combined with a 13 bp insertion at the beginning of exon 2, introducing a stop codon. The seven other nonsense mutations substitute a stop codon for the wild type amino acid (table 3). Finally, five splice site mutations were found: a C→G substitution 3 bp before the acceptor splice site in intron 27 (patient 6); an A→T substitution 1 bp after the donor splice site in intron 19 (patient 12); a T→C substitution of the GT donor splice site in intron 25 (patient 66), and a 7 bp deletion together with a 2 bp insertion at the donor splice site of intron 17 (patient 44). In patient 65, a missense mutation (K1521R) was found in exon 27, 2 bp before the donor splice site. Analysis of this mutation with the Splice Site Prediction by Neural Network software showed that the corresponding A→G substitution suppressed the donor site. Analysis of the splice mutations found at the donor sites in patients 44, 66, and 65 with this software indicated that all of them suppressed the donor site (predicted scores = 0%) and created a premature stop codon, potentially leading to a truncated protein. For patient 12, analysis of the splice site mutation showed that the donor site prediction score decreased from 100% to 70%, suggesting that this mutation was responsible for a leaky splicing process. Analysis of the single acceptor site mutation found in our series (patient 6) indicated the suppression of the acceptor site, leading to exon 28 skipping. However, the open reading frame is maintained between exons 27 and 29, creating a protein lacking 56 amino acids. All these changes are potentially pathological. However, since no source of RNA was available for the patients displaying these splice site mutations, a more detailed study by RT-PCR or northern blot analysis could not be performed.

Careful examination of the patients' phenotypes indicated that all patients expressed the RTS phenotype including broad thumbs or halluces, facial dysmorphism, and mental retardation. Thirty patients (47.6 %) were found to have a *CBP* abnormality (gross rearrangement, RT100 deletion, *CBP* missense, nonsense, or splice site mutation). In 33 cases, no abnormality was detected. Comparing the phenotypes of patients with and without a mutation may help in establishing genotype-phenotype correlations, especially with respect to classifying patients into subgroups. Comparison of the phenotypes of patients with and without *CBP* deletions has previously been reported.[10 12] In table 4, we focus on the comparison of phenotypes of patients with or without a presumably deleterious effect, that is, a nonsense mutation or a splice site mutation leading to a stop codon. Patients with a missense mutation were not included, since their effect on *CBP* function is more difficult to quantify. Complete clinical documentation was not available for all patients, as is often the case for such complex syndromes as Rubinstein-Taybi syndrome. For instance, mental retardation could not be assessed in newborns, but was

Table 2 Oligonucleotides and PCR conditions for amplification and direct sequencing of *CBP* exons

CBP exon	Position*	Fragment size (bp)	Primer name	Primer sequence 5′→3′	PCR conditions $MgCl_2$ - Annealing T
Exon 1 (890 bp)	–22, + 95	200	EX1-L	CTGTTTTCGCGAGCAGGT	2 mmol/l - 50°C
			EX1-R	ATCGGTATCCGCGACCAC	
Exon 2 (713 bp)	–41, +44	798	EX2-L	GGTTGCTTAGTTTCTCATTTCCA	2 mmol/l - 55°C
	+227		EX2-R	CCGCGGTTAGGTAGGAAGTA	
	+253		EX2-L1	GGGCAGCCGAACAGTGCTAACATGGCC	
	+456		EX2-R1	GGCCATGTTAGCACTGTTCGGCTGCCC	
	+477		EX2-L2	TTAACCAGACCCAGGCC	
			EX2-R2	GGCCTGGGTCTGGTTAA	
Exon 3 (177 bp)	–64, +43	284	EX3-L	CCCAGGCCCTTCCTTTTAT	2 mmol/l - 55°C
			EX3-R	ACCCACCGGAGAGCCATA	
Exon 4 (241 bp)	–87, +41	369	EX4-L	AGCTTTTGCTTTTGTTTGAGA	2.5 mmol/l - 55°C
			EX4-R	TCCTGACCTCTACCACTAGGAG	
Exon 5 (114 bp)	–57, +87	258	EX5-L	TTGAAAGTGTGACGATTTGGA	2 mmol/l - 60°C
			EX5-R	TGCCCACTCCCTACCTACTC	
Exon 6 (243 bp)	–61, +50	354	EX6-L	CGTGGGCTTCTCCCTTTTAC	2 mmol/l -55°C
			EX6-R	TTTGCTGCATGTGGACAAGT	
Exon 7 (103 bp)	–86, +43	232	EX7-L	TGGTGGCATGTTGGTTATCT	2 mmol/l - 55°C
			EX7-R	TGCTCCTGTTGGACTGTCAC	
Exon 8 (147 bp)	–83, +58	288	EX8-L	TGTGGTGGCAGAAGAACCTT	2 mmol/l - 55°C
			EX8-R	CAATGGGCAACACAGGAATA	
Exon 9 (118 bp)	–71, +81	273	EX9-L	GGACGAAACCCCATGTCTTT	2 mmol/l - 55°C
			EX9-R	TTCCAGATAACAAAGCAGACAAA	
Exon 10 (172 bp)	–67, +37	276	EX10-L	CAACACAGATCATTCAGTTGCTT	2 mmol/l - 55°C
			EX10-R	CAGGCTAAGGGATGGCAGTA	
Exon 11 (45 bp)	–81, +63	189	EX11-L	AAGAAATTTCCTATTCCTGAATCAA	2 mmol/l - 55°C
			EX11-R	CAGTGAAAGTTATGGCTGTTGAA	
Exon 12 (125 bp)	–45, +25	195	EX12-L	CCTGTGGGTGCTTTTCCTAA	2 mmol/l - 55°C
			EX12-R	CAGCACAGCGAAAGAGAACA	
Exon 13 (180 bp)	–46, +66	292	EX13-L	CGTCAGCTTCCGAACTACAG	2 mmol/l - 55°C
			EX13-R	AAAAACCAAAACTTAACACAAGAATTT	
Exon 14 (417 bp)	–49, +39	505	EX14-L	TTTTCAAACGGGGGAAATAA	2 mmol/l - 55°C
	+190		EX14-R	GAGTCTTGGCCCAAAAACAG	
	+209		EX14-L1	CCACTCAGCCATCAACTCCT	
			EX14-R1	AGGAGTTGATGGCTGAGTGG	
Exon 15 (180 bp)	–74, +43	297	EX15-L	TGCATGAGCAGCATAGTTGA	2 mmol/l - 55°C
			EX15-R	GTTGCGATACGCAGTAATG	
Exon 16 (190 bp)	–74, +56	320	EX16-L	AGAGTCTTCCCGTGAGGTTG	2 mmol/l - 55°C
			EX16-R	TCCTAACACCGTGGAAAAGC	
Exon 17 (119 bp)	–93, +53	265	EX17-L	ATGTCACAGCAGCCAGGATT	1.5 mmol/l - 60°C
			EX17-R	CACTCAGAAGTCACACCAGCA	
Exon 18 (240 bp)	–67, +58	365	EX18-L	GCCAGATGAGACTGGCATTT	2 mmol/l - 55°C
			EX18-R	ACCCCTCTGGCTGGATTAAC	
Exon 19 (89 bp)	–65, +47	201	EX19-L	GAACATTATAAGACAGTAAATGGAATG	2 mmol/l - 55°C
			EX19-R	ACGTGCCTTGCCCTAAGAC	
Exon 20 (81 bp)	–59, +50	190	EX20-L	TTGGGTGGCTGTGTGTTATG	2 mmol/l - 55°C
			EX20-R	TTTAAGGTCACCCTCCCTCA	
Exon 21 (57 bp)	–85, +50	192	EX21-L	CAAAATAACATTCCAGAGACCCTA	2 mmol/l - 55°C
			EX21-R	CCGATTTCAAACCAAAACTGA	
Exon 22 (78 bp)	–81, +87	246	EX22-L	GGACGCACACACAGACTTCTAC	2 mmol/l - 55°C
			EX22-R	ACAATGAATGAGATGCAGTAGCC	
Exon 23 (68 bp)	–67, +65	200	EX23-L	TGCATTTTGTTGGTTTGACAAT	2 mmol/l - 55°C
			EX23-R	GGGGACAATTTCTACAAGTTTCTAA	
Exon 24 (151 bp)	–42, +81	274	EX24-L	TGCTGTTGAAGCCCTCTCAC	1.5 mmol/l - 60°C
			EX24-R	CAAGAGCTTTGCAGAGAGCA	
Exon 25 (147 bp)	–66, +45	258	EX25-L	CTGGTGTGCAGAAGCACCT	1.5 mmol/l - 60°C
			EX25-R	CACGGCTCACTGAATGACAC	
Exon 26 (114 bp)	–81, +113	308	EX26-L	TTCCAGGGTGTTGTTTGTTG	1.5 mmol/l - 60°C
			EX26-R	GGATGGAAAAATAAAAACGCATA	
Exon 27 (166 bp)	–61, +74	301	EX27-L	CTTAAAGGCAGGGCCGATT	1.5 mmol/l - 60°C
			EX27-R	TGCAAGAAAAAGGCACACAA	
Exon 28 (168 bp)	–122, +37	327	EX28-L	CACACATGCATGGGACTCTG	1.5 mmol/l - 60°C
			EX28-R	GACACGTGGGCAATGGAG	
Exon 29 (162 bp)	–36, +60	258	EX29-L	ACTTGCCTGGTCTCACAGC	1.5 mmol/l - 60°C
			EX29-R	TGCGAGTCTTTCCCTCCTC	
Exon 30 (282 bp)	–55, +60	397	EX30-L	ACCACTGGAGGTGCCATGT	1.5 mmol/l - 60°C
			EX30-R	ACAGGATGCTTCGTCAGACC	
Exon 31 (3077 bp)	+708, +1534	826	EX31-L2	GCAGATCGAGCGTGAG	1.5 mmol/l - 60°C-
	+1372, +2285	913	EX31-R4	CTTGAGGCTGCTGGAACTG	2.5% formamide
	+1074		EX31-L4	TGGCGAGTATGAATCCACAG	1.5 mmol/l - 55°C
	+1166		EX31-R	ACATCAATCCACCCTTC	
	+1833		EX31-L3	CAACAGCAGGTGCTGAACAT	
	+1848		EX31-R3	GGCTGATTGGCCACGTACT	
			EX31-L5	GCCACGTCCCTTAGTAACCA	
			EX31-R5	ACTAAGGGACGTGGCGATCT	

Column 1 gives the exon number and length in bp.
*Column 2 corresponds to the relative position of left and right primers relative to the beginning and the end of the exon respectively, except for exon 31 for which all positions are given relative to the beginning of the exon.

Table 3 Mutations in the *CBP* gene detected by PCR and direct sequencing

Patient No	Mutation location	Mutation type	Protein domain*
Missense mutations			
29	Exon 15	A981T (GCA→ACA)	
30	Exon 31	N1978S (AAC→AGC)	GR
58	Exon 31	M2221L (ATG→CTG)	GR
50	Exon 31	A2243V (GCC→GTC)	GR
Nonsense mutations			
69	Exon 1	S23X (TCG→TAG)	NRBD
40	Exon 4	R370X (CGA→TGA)	NTD
75	Exon 5	R413X (CGA→TGA)	NTD
18	Exon 21	K1269X (AAG→TAG)	HATD
35	Exon 27	R1498X (CGA→TGA)	HATD
64	Exon 27	Y1466X (TAT→TAA)	HATD
57	Exon 31	Q2043X (CAG→TAG)	CTD
8	Exon 14	2827delC	
39	Exon 30	4945delA	E1ABD
52	Exon 2	138delA+ins13bp†	NRBD
68	Exon 3	840insT	NTD
51	Exon 10	2045insA	CBD
33	Exon 16	3096insT	
Splice site mutations			
6	Intron 27	IVS27–5C→G	HATD
44	Intron 17	del7bp+ins2bp‡	
12	Intron 19	IVS19+3A→T	HATD
66	Intron 25	IVS25+2T→C	HATD
65	Exon 27	K1520R§ (AAG→AGG)	

*Last column refers to numbers of the different fuctional domains of *CBP* given by Giles *et al.*[9] NRBD: nuclear receptor binding domain; NTD: N-terminal transactivation domain; CBD: CREB binding domain; HATD: histone acetyltransferase domain; E1ABD: E1A binding domain - 3rd Cys/His rich region - 2nd zinc finger; GR: Gln rich region; CTD: C-terminal transactivation domain. Some of the mutations are not related to any protein domain since certain regions of the *CBP* gene have not been assigned to any functional domain.
†Normal sequence ATACCCAATGGAGGAGAA.
Mutated sequence: ATACCC - TCATCATGAGCTGATGGAGGAGAA.
‡Normal sequence: ATCCA*GT*AAGTTAATTCAT.
Mutated sequence : ATCCCA - - - - - TAATTCAT.
§Missense mutation affecting the donor splice site (see Results section).

observed in all patients who were above 6 months at the time of diagnosis. No striking phenotypic difference could be observed between the two groups of patients. However, a highly arched palate, which is a well known sign of Rubinstein-Taybi syndrome, was found in 15/16 patients with a mutation and only in 15/26 of those without a mutation. Also interesting is the fact that none of the patients (0/16) with a mutation showed a valgus deviation of the halluces, whereas 6/24 patients without a mutation had one. Although the number of patients analysed was small, these observations deserve closer attention in further studies of patients with Rubinstein-Taybi syndrome.

Table 4 Phenotype comparison between non-deleted, non-mutated patients and patients with a deleterious *CBP* mutation

	Patients without mutation or deletion (%)	Patients with a stop or splice mutation (%)
Prominent or beaked nose	75.0 (21/28)	88.8 (16/18)
Highly arched palate	57.7 (15/26)	93.7 (15/16)
Downward slanting palpebral fissures	82.6 (19/23)	94.1 (16/17)
Broad thumbs	85.7 (24/28)	94.1 (16/17)
Radially deviated thumbs	52.0 (13/25)	58.8 (10/17)
Broad halluces	83.3 (25/30)	100 (17/17)
Valgus deviation	25.0 (6/24)	0 (0/16)
Varus deviation	12.5 (3/24)	31.2 (5/16)
Growth retardation	91.3 (21/23)	93.7 (15/16)
Mental/psychomotor retardation	100 (26/26)	100 (15/15)
Hypotonia	72.0 (18/25)	62.5 (10/16)
Microcephaly	65.4 (17/26)	68.7 (11/16)

No significant abnormality was obtained with χ^2 test (all values under 2.5).

DISCUSSION

We first refined the physical map of the cytogenetic band 16p13.3 region around *CBP* by properly ordering the microsatellite markers and by identifying three novel intragenic microsatellites. For one patient (59), we confirmed a deletion found by FISH with the RT100 probe and evaluated the extent of the deletion. The deletion was framed by markers D16S3134 on the proximal side and D16S2622 on the distal side, and covered at least 560 kb. Analysis of intragenic microsatellite markers in another patient (34), who was found to be deleted for RT100, showed that this patient was heterozygous for D16S3065, suggesting a partial deletion of the 3′ end of the *CBP* gene. On the other hand, analysis of intragenic markers in patient 9 indicated a partial deletion of the 5′ end of the *CBP* gene.

These results showed that analysis of the four intragenic microsatellite markers was a useful way to test patients for microdeletions and could represent a first step of screening before FISH analysis is undertaken. Indeed, patients found to be heterozygous for all intragenic markers could be excluded from an exhaustive FISH analysis requiring the use of five cosmid probes.[8] From this point of view, it is interesting to note that the analysis of 50 controls showed that 14 (28%) were heterozygous for all four intragenic markers and 20 (40%) were heterozygous for three markers. FISH analysis will remain necessary with AC004651 and AC004760 to avoid missing deletions that are restricted to the 3′ end of the gene, and possibly with AC004509 to exclude an interstitial deletion between MS2 and D16S3065.

Marker MS4 is close to the highly unstable region of the *CBP* gene already identified by others around exon 2 and containing the chromosome 16 breakpoints of chromosomal rearrangements found in RTS and leukaemias, suggesting inherent instability.[9] Analysis of the high throughput genome

sequence data identified a BAC clone (AC007151) that contained the 5′ end of the *CBP* gene (exon 1 to exon 3). BLAST analysis of this sequence allowed us to determine the exact size of intron 2 (39 517 bp), which was not previously known. In addition, we were able to show that the gap remaining in the sequence between cosmids RT203 (LANL cosmid 400H11 or AC004495) and RT166 (LANL cosmid 420F6 or AC005564)[13] was now filled and spanned 11 173 bp. A NIX analysis focused on the unstable region of *CBP* indicated the presence of a total of 57% interspersed repeats in this region (27.7% of SINEs, 17.8% of LINEs, 0.8% of LTR elements, and 10.7% of MER elements). In comparison, the other two cosmids covering the 3′ part of the *CBP* gene (AC004509 and AC004651) presented a total of 28.6% and 35.3% interspersed repeats respectively. The high density of repeat elements observed in the 5′ region of the *CBP* gene could be responsible for the observed instability leading to numerous chromosomal rearrangements.

Sixty-three patients were included in the present study. Three microdeletions were detected by FISH analysis with the RT100 probe, three gross rearrangements were found by Southern blot and/or microsatellite analysis, one small intragenic deletion was found by RT-PCR, and one truncated RNA was detected by northern blot analysis. Twenty-two point mutations were identified by direct sequencing, representing 36.7% of the 60 RTS cases included in the sequencing study. None of these mutations was described in previous reports.[8 9] Parental DNA was available for only eight patients. Two of them (9 and 59) were carrying a *CBP* deletion analysed with microsatellite markers and patients 38 and 49 were neither deleted nor mutated. Patients 40, 51, 52, and 69 have been found to have mutations (four nonsense mutations). The de novo status of these mutations could be inferred because they were not found in both parents. For all other patients, parental DNA was not available. However, since RTS is an autosomal dominant trait, it can be assumed that the nonsense mutations found occurred de novo. Concerning the four missense mutations, since they were found only once among the 60 patients analysed (that is, 120 independent chromosomes), it can be assumed that they represent true mutations and not polymorphisms. Moreover, when compared with the murine *CBP* (Genbank Accession No S66385), which shows 91% identity over the whole 2441 aa of the protein, all of the mutated amino acids were conserved, suggesting the functional importance of these amino acids in human *CBP*.

There were no significant differences in the clinical manifestations of RTS patients with or without CBP mutations. Bartsch *et al*[12] suggested a more severe RTS phenotype characterised by visceral abnormalities (hypoplastic left heart, abnormal pulmonary lobulation, polysplenia) and early death in some patients with large deletions. Patient 59 in our series has a large deletion covering at least 560 kb between flanking markers D16S3134 and D16S2622, and has severe growth and mental retardation (IQ 20-30), significant keloids, and Hodgkin's lymphoma. This deletion removed not only the entire *CBP* gene but also the following genes (according to GenBank contig NM_015360): *ADCY9* (adenylate cyclase type 9, Acc No AF036927), *HMOX2* (haem oxygenase 2, Acc No AF051306), *LOC94389* (similar to *Drosophila* tumorous imaginal discs homologue, Acc No AF061749), *GLIS2* (Kruppel-like zinc finger protein, Acc No AF325914), and *TFAP4* (transcription factor AP-4, Acc No NM003223). Some of these genes are potential tumour suppressor genes, the deletion of which might affect the phenotype, for example, a predisposition to develop tumours. This patient who is now 30 years old therefore has a severe "classical" RTS phenotype, but does not have any of the particular clinical manifestations described by Bartsch *et al*[12] such as a hypoplastic left heart. Therefore, although large deletions may account for the more severe forms of the disease, they are not necessarily associated with early death and profound disturbance of laterality. It may be that the deletions described by Bartsch *et al*[12] were larger than the one present in patient 59 and remove additional genes that are important for heart development.

Taken together, our results showed that a combination of the various techniques enabled us to identify a *CBP* mutation in 47.6% of RTS cases, which represents almost 2.5 as many as any results reported so far. In the present study, no *CBP* abnormalities were found in 33 RTS patients. However, intragenic microsatellite markers and/or FISH analysis with the four other cosmid probes are still under investigation and could lead to the finding of more deleted patients. Preliminary results showed that only one allele could be detected at all intragenic microsatellites in four patients. Although these data did not allow us to discriminate between hemi- or homozygous status at this point, they suggest that these patients might carry a deletion. These results will be confirmed by FISH analysis. Other mutational events could involve the *CBP* promoter (direct sequencing of the 5′ end of the *CBP* gene has not been achieved so far), intronic mutations that affect transcription, or silent mutations in exons. Silent mutations inducing exon skipping have been reported in Marfan syndrome (MFS, MIM 154700) and ataxia-telangiectasia (AT, MIM 208900).[14 15] Finally, the wide range of phenotypes in RTS patients and the dearth of *CBP* mutations in some patients suggest that not only the loss of one functional copy of the *CBP* gene but also mutations in other genes, such as the *CBP* homologue p300 or genes encoding for proteins that interact with *CBP* in various signal transduction pathways, may play a role in the aetiology of RTS.

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Electronic database information. Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Rubinstein-Taybi syndrome (MIM 180849), for Marfan syndrome (MIM 154700)) and ataxia-telangiectasia (MIM 208900)). Primer 3 Output software, www.genome.wi.mit.edu//cgi-bin/primer/primer3_www.cgi. BLAST search, http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast. Splice Site Prediction by Neural Network software, http://www.fruitfly.org/seq_tools/splice.html (for NIX analysis). Genbank, http://www.ncbi.nlm.nih.gov/Web/genbank/ (for CBP mRNA sequence (U85962); for cosmids and BAC sequences (AC005564), (AC007151), (AC004495), (AC004509), (AC004651), (AC004760)). CEPH-Généthon Integrated Map, http://www.cephb.fr/ceph-genethon-map.html/. Marshfield Center for Human Genetics, http://www.marshmed.org/genetics/ (for microsatellite markers information).

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ECHO

MICA-A5.1 weighs in with HLA-DR3/DQ2 in coeliac disease

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People with atypical forms of coeliac disease (CD) have another gene which may influence their disease as well as the already recognised MHC DR3/DQ2 allele. MHC class I chain related gene A (*MICA*), which specifies an enterocyte protein recognised by T cells and exists in strong linkage disequilibrium with HLA-B, is involved in atypical CD.

Among 133 patients with CD the frequencies of the heterodimer DQA1*0501/DQB1*0201 were similar in patients with typical or atypical CD and much higher than in the controls, as expected. Patients with atypical CD, however, had a significantly greater frequency of the extended haplotype E8.1 (B8/DR3/DQ2) (odds ratio 4.19; 95% confidence interval 1.97 to 8.84) than those with typical CD or the controls, and also of polymorphism *MICA-A5.1* (odds ratio 8.63; 3.11 to 23.94).

This relation could not be explained by the linkage disequilibrium known to exist between *MICA-A5.1* and extended haplotype E8.1. Although all patients and controls with the extended haplotype E8.1 also had the *MICA-A5.1* allele, patients without this haplotype had a significant overrepresentation of the *MICA-A5* allele if they had atypical CD (64% (9/14) *v* 22% (10/47), odds ratio 6.66; 1.82 to 24.36).

The study included 133 consecutive Spanish patients with CD presenting to two hospitals, 79 with typical and 54 with atypical CD according to clinical symptoms, and 116 healthy unrelated controls from the general population. Each was typed for HLA-DRB1, DQA1, DQB1 and exon 5 of the *MICA* gene by polymerase chain reaction sequence specific primers.

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