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Use of a set of highly polymorphic minisatellite probes for the identification of cryptic 1p36.3 deletions in a large collection of patients with idiopathic mental retardation

EDITOR—Mental retardation is a component of a large number of syndromes, most of which qualify individually as rare genetic diseases. Altogether, mental retardation affects 2-3% of the population and is unexplained in 40% of cases. According to Knight *et al.*,¹ subtle telomeric chromosomal rearrangements are responsible for approximately 1% of unexplained mental retardation (with the proportion being highest, 7.4%, in the subclass of unexplained moderate to severe mental retardation). The identification of the genes responsible will require the precise delimitation of minimum deletion regions, which relies upon the collection of a large number of cases. Because of the low expected frequency of each telomeric deletion in mental retardation, the procedure to be applied should allow the screening of many patients at a low cost.

It is not yet clear whether all chromosome ends are associated with mental retardation syndromes with similar frequency. Distal chromosome 1p36 deletions were initially detected cytogenetically because of an associated segmental imbalance.²⁻⁵ This syndrome results from both interstitial and terminal deletions of varying sizes and different breakpoints⁶ and the severity of the phenotype is related to the extent of the deletion.⁷ Clinical examination can efficiently detect a large proportion of cases, so that the number of reports of 1p36 deletions has increased significantly in the past few years which may give the false impression that this is a relatively frequent syndrome.

We show here how highly polymorphic minisatellites located within a short region can provide an efficient pre-screening of samples without the need for parental samples at the initial stage. The procedure was tested here using 1p36 minisatellites and provides an estimate of the frequency of 1p36 deletions in mentally retarded patients. A selection of five highly polymorphic minisatellite probes was used to search for 1p36.3 deletions in a collection of 567 patients with mental retardation. Three patients showing a single allele at all five loci were identified and in each case segmental 1p36 aneusomy was confirmed by *in situ* hybridisation. The physical extent of each deletion was determined by analysis of additional markers. In two cases, the parental origin of the deletion could be determined, one is maternal and the other is paternal. Analysis of band intensity of samples showing a single allele at two or more contiguous minisatellite loci ruled out the possibility of shorter deletion in this collection of patients. These data and earlier investigations suggest that 1p36.3 deletions account for as much as 0.5-0.7% (96% confidence interval 0.15-1.2%) of idiopathic mental retardation.

All participating families gave informed consent to the search for a genetic abnormality which might explain the condition of their child. Most DNA samples (469/567) were initially collected and extracted in the course of a search for the fragile X. All patients showed a normal routine karyotype.

DNA was extracted from blood collected on EDTA essentially as described by Jeanpierre.⁸ Five µg of DNA

were digested with *AluI* (Boehringer Mannheim), electrophoresed through a 1% agarose gel, and transferred to nylon membranes (Nytran+, Schleicher and Shuell) under vacuum (Pharmacia Biotech).

Minisatellite DNA inserts were [³²P]dCTP labelled by random priming⁹ and hybridisation was done at 65°C as previously described.¹⁰ The minisatellites used in this study (CEB66, B4, CEB42, CEB108, CEB15, CEB88, CEB37, UPS2, CEB20, CEB55, and CEB121) were isolated essentially as previously described.¹⁰⁻¹³ CEB66, B4, and CEB42 minisatellites are located at 13q34, Xpter, and 8q24 respectively. CEB66 and B4 are monomorphic and were used when necessary as controls for DNA quantification. The highly polymorphic CEB42 probe was used to confirm parentage. The other loci have been previously assigned to 1p36.3 by FISH and segregation analysis.¹²⁻¹⁵ All probes used in this study have been used in linkage mapping in the CEPH families and no evidence for *de novo* deletion, trisomy, or UPD in the DNA extracted from lymphoblastoid cell lines (more than 500 subjects) was found.

Microsatellites were purchased from Research Genetics. One primer was kinased with γ [³³P]dATP (Isotopchim) as recommended by the manufacturer (kinase from New England Biolabs). PCR reactions were performed as previously described¹⁶ using a Perkin-Elmer GeneAmp PCR system 9600. PCR products were separated on 6% polyacrylamide/urea gels. The gel was dried after transfer onto a Whatman 3MM paper and exposed to an RX Fuji film.

Segregation data in the CEPH families were managed using the GENBASE software developed by Jean-Marc Sebaoun at CEPH.¹⁷ Analyses were done using the CRIMAP version 2.4 package.¹⁸ Minisatellites were typed on the 40 CEPH families by Southern blotting. Meiotic breakpoint analysis was further assisted by the software described by Attwood and Povey.¹⁹ Microsatellite genotyping data on the eight CEPH families were recovered from the CEPH database at <http://www.cephb.fr>. Microsatellite typings on the remaining 32 CEPH families were done to increase the mapping resolution. In order to reduce typing effort, only key recombinant subjects were typed, together with the two parents and appropriate grandparents as outlined in Cox *et al.*²⁰ All genotyping data generated in the course of this project can be recovered from CEPH at <http://www.cephb.fr/>.

Chromosome preparations were made from peripheral blood samples or lymphoblast cultures by conventional methods. Karyotyping with RBG banding was performed at the 550-850 band level according to standard methods. Alternatively, high resolution R banding was obtained by introduction of 5-bromodeoxyuridine (BrdU) for 5½ hours in cell cultures after thymidine synchronisation.²¹

Cosmid DNA was labelled with digoxigenin 11dUTP using the Dig-Nick translation kit from Boehringer Mannheim Inc. One hundred ng of the resulting digoxigeninylated DNA were preannealed with 2.5 µg of *cot I* DNA (Gibco BRL) in a total volume of 10 µl hybridisation mix (50% formamide, 2 × SSC, 10% dextran sulphate) for 30 minutes at 37°C. Hybridisation to the previously denatured chromosomes was done overnight at 37°C. Post hybridisation wash was carried out at 72°C in 2 × SSC for five minutes. Probe detection was performed with antidigoxin conjugated fluorescein isothiocyanate (FITC, Sigma). Slides were mounted in antifade solution (Vectashield, Vector Laboratories) containing 4', 6-diamidino-2-phenyl indole (DAPI) as a counterstain.

Table 1 Integrated linkage map and extent of the deletion in the three patients

Marker name	D number (accession number)	Het (%) CEPH families	Physical distance from telomere (in centirads)	Genetic distance from telomere (in centimorgans)	Patient 1	Patient 2	Patient 3
<i>CEB108</i>	NA	94		0.2			
<i>CEB15</i>	D1S172 (AL096805)	100		0.8			
<i>AFM214yg7</i>	D1S243	86	13.58	2.6			
p1-79	D1Z2	70		2.6			
<i>CEB88</i>	D1S338	97		6.3			
<i>CEB37</i>	D1S337	87		7.2			
<i>UPS2</i>	D1S337	75		7.4			
<i>AFM280we5</i>	D1S468	75	16.94	7.8			
<i>CEB20</i>	D1S173	75		7.8			
<i>CEB55</i>	D1F101S2	59		10	D		
<i>AFM344we9</i>	D1S2845	84		11.1	NI		
<i>AFMa203yc1</i>	D1S2660	78		12.8	Normal		
<i>CEB121</i>	NA	77		14.2		D	
<i>FO525</i>	D1S2694 (AJ001151)	78		17.4		Normal	D

D=deleted. NI=not informative. Normal=not deleted. NA=none assigned. The five highly polymorphic minisatellites used in the screening are italicised. *CEB121* and Genethon microsatellite markers *D1S2795*, *D1S2633*, and *D1S2145*¹⁶ cannot be ordered by linkage analysis in the 40 CEPH families. *FO525* (accession number AJ001151) is physically linked to Genethon marker *D1S2694*.

Slides were analysed using a Zeiss Axioskop microscope equipped with a Pinkel filter set for visualisation of FITC and DAPI fluorescence. Digital images were recorded with a Photometrics cooled CCD camera. Pseudo colouring and merging of image were done with the Mac Probe 3.3 software.

Because most markers used here are highly polymorphic and because genetic distances are usually largely inflated towards chromosome ends (in the order of 100 kilobases/cM ratio in male meiosis), linkage mapping using the CEPH families panel was used as an efficient way of ordering the loci and inserting them into the Genethon microsatellite reference map.¹⁶ Table 1 shows the deduced integrated map. Almost all minisatellites used here surround the two most distal markers on the Genethon map (*D1S243* and *D1S468*). A single RPCI6 PAC contains both *CEB88* and *CEB37*, and *UPS2* was recovered from a PAC positive for *CEB37* (data not shown). Consequently, it is very likely that loci unseparated by crossover in the 40 CEPH families (and listed in an arbitrary order in table 1, *D1S243* and *D1Z2*, *D1S468* and *CEB20*) are separated by less than a few tens of kilobases. The radiation hybrid mapping data available at <http://www.sanger.ac.uk> suggest a 13 cR distance between *D1S243* and *D1S2694* (out of a total of 818 cR and 263 Mb for chromosome 1), which would then roughly convert into a maximum estimate of 4.2 Mb for the *D1S243*-*D1S2694* interval (including 1 Mb for the *D1S243*-*D1S468* interval).

DNA samples from 567 patients were screened by Southern blot analysis using five minisatellite probes (*CEB108*, *CEB15*, *CEB88*, *CEB37*, *UPS2*). Of these, 20.3%, 11.6%, 7.2%, 32.8%, and 21.9% of patients had only one allele at *CEB108*, *CEB15*, *CEB88*, *CEB37*, and *UPS2* loci, respectively (table 2). Sixteen (2.8%), seven (1.2%), 20 (3.5%), and 42 (7.4%) patients displayed a single band at the pairs of contiguous loci *CEB108/CEB15*, *CEB15/CEB88*, *CEB88/CEB37*, and *CEB37/UPS2*, respectively. Four (0.7%), six (1%), and nine (1.6%) patients displayed a single band for three adjacent probes (*CEB108/CEB15/CEB37*, *CEB15/CEB88/CEB37*, and *CEB88/CEB37/UPS2* respectively). Three patients showed a single allele at *CEB108/CEB15/CEB88/CEB37* and four at *CEB15/CEB88/CEB37/UPS2*. Three patients (0.5%) showed a single allele at all five loci. Band intensities in subjects showing a single allele for at least two contiguous loci were then analysed (data not shown). This required that the amount of DNA loaded in each lane was quantified precisely. For this purpose, two monomorphic minisatellites derived from other chromosomes were used. *CEB66* is a minisatellite from 13q34 giving a 2.9 kb allele on *AluI* digests of human DNA samples. *B4* is derived from

the XpYp pseudoautosomal region and gives a constant 1.2 kilobase *AluI* fragment.¹¹ This analysis indicated that only the three patients showing a single band for all five loci are likely to be hemizygous. The deletion was confirmed in all three cases by FISH. Patient 2 is deleted for *CEB108*, *CEB15*, *CEB88*, *CEB55*, and *CEB121* (14.2 cM) and not deleted for *FO525* (17.4 cM). The minisatellite alleles of patient 2 corresponded to one of the paternal alleles, suggesting that the deletion is of maternal origin (DNA sample from the mother was not available). Patient 3 is deleted for *CEB121* (cosmid *Icrf c112-J06107*) and for cosmid *F0525* (locus *D1S2694*) (table 1) and has the largest deletion. The parents could not be studied in this case.

Owing to the accessibility of DNA from the parents, the deletion origin and size of patient 1 could be analysed in more detail. The deletion is a de novo deletion of paternal origin and ends between *CEB55* (10 cM) and *D1S2660* (12.8 cM) (table 1).

Patient 1 is the second child of unrelated parents with no particular family history. She was born at 41 weeks of gestation after an uneventful pregnancy. She weighed 3060 g; her length and OFC were 49.5 cm and 34 cm, respectively. The first months of life were marked by severe hypotonia. Development and growth were delayed; she sat at 10 months and walked at 30 months. At 3½ years, there was moderate growth delay; weight, length, and OFC were 10.3 kg (-3 SD), 93 cm (-1 SD), and 47 cm (-2 SD), respectively. Mild facial dysmorphic features were noted, including a triangular face, pointed chin, flat nasal bridge, broad forehead, small, low set, and posteriorly rotated ears, thin, horizontal palpebral fissures, deep set eyes, moderate epicanthic folds, and convergent strabismus. Eye examination was normal (fig 1A, B). Small fifth digits and hallux valgus were noticed. No other visceral malformation was found. Mental retardation was severe and at 3½ years, her development was equivalent to 18 months with a total lack of speech but without impairment of hearing or vision. She

Table 2 Number and frequency of subjects showing a single allele for different combinations of minisatellites. The probes are ordered from the most distal (*CEB108*) to the most proximal (*UPS2*). The values along the diagonal indicate the total number of subjects showing a single band (and percentage) for each probe. The values given below the diagonal indicate the number of samples showing a single band for all probes in the interval (for example, 4 in the *CEB108-CEB88* cell indicates that four DNA samples show a single band for *CEB108*, *CEB15*, and *CEB88*). The values given in brackets indicate the corresponding percentage

Probes	<i>Ceb108</i>	<i>Ceb15</i>	<i>Ceb88</i>	<i>Ceb37</i>	<i>UPS2</i>
<i>Ceb108</i>	115 (20.3%)	2.8%	0.7%	0.52%	0.52%
<i>Ceb15</i>	16	66 (11.6%)	7%	1%	0.7%
<i>Ceb88</i>	4	7	41 (7.2%)	1.2%	1.6%
<i>Ceb37</i>	3	6	20	186 (32.8%)	3.5%
<i>UPS2</i>	3	4	9	42	124 (21.9%)

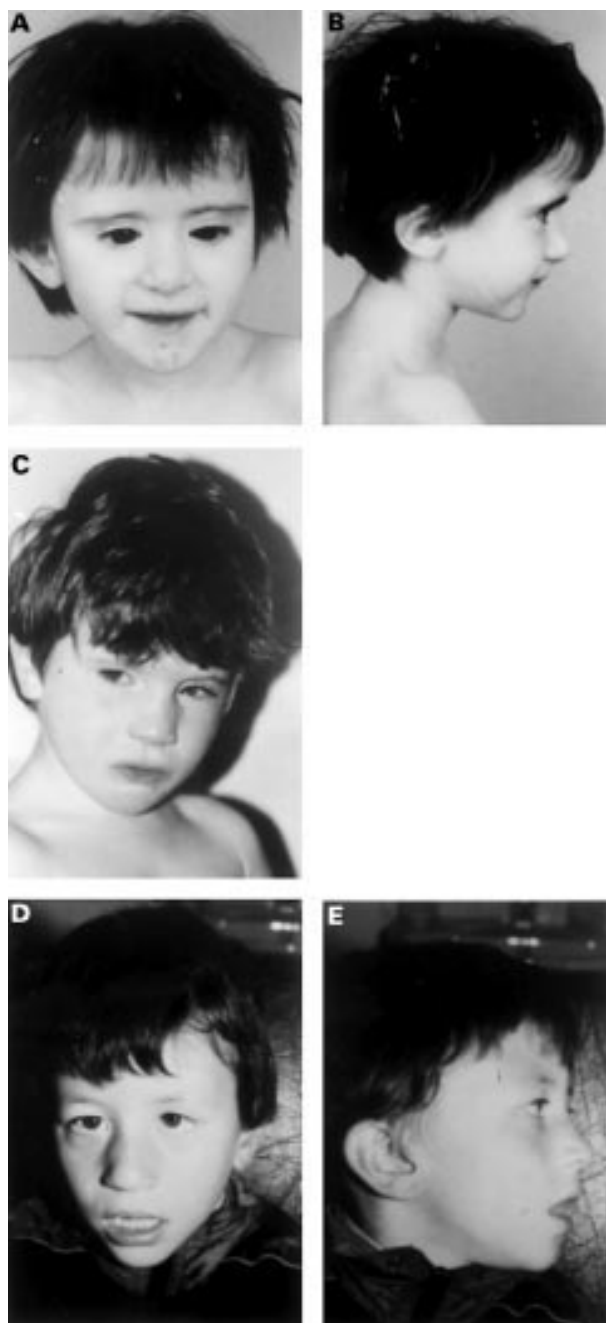


Figure 1 (A, B) Patient 1 aged 4 years. (C) Patient 2 aged 5½ years. (D, E) Patient 3 aged 5 years.

spoke her first words at 4½ years and said two words together at around 5½ years. There was no self-mutilation or behavioural difficulties. Magnetic resonance imaging of the brain was normal.

Patient 2 was referred at 5 years for evaluation of mental retardation. He was born to a healthy, non-consanguineous 17 year old mother and 21 year old father at 42 weeks of gestation. The mother has a normal boy from another union. Family history and pregnancy were unremarkable. Apgar score was 10/10. At birth, weight was 3540 g, height 50 cm, and OFC 37 cm. As a neonate, he was noted to have an unusual facial appearance with frontal bossing and a large anterior fontanelle (closed at 2½ years). He had myoclonic seizures in the neonatal period. The patient had severe developmental delay with hypotonia, he sat at 2 years and stood at 3 years, and had no acquisition of language. Behavioural problems included waking during

the night, self-injurious manifestations, and autistic behaviour. He had postnatal growth retardation with a weight of 12.3 kg at 4 years and 16 kg at 5.5 years (10th centile) and a height of 90 cm at 4 years and 100 cm at 5½ years (<3rd centile). On physical examination, he had facial dysmorphism with a narrow forehead, deep set eyes, high nasal bridge, long philtrum, everted lips, low set ears with thickened helices, and a pointed chin (fig 1C). OFC was 49 cm (3rd centile). The hands and feet were short with 5th finger clinodactyly and overlapping toes. He had scrotal hypoplasia and divergent strabismus with a left iris coloboma and chorioretinal atrophy. Audiological testing and cardiac ultrasound were normal. Brain MRI showed mildly enlarged lateral ventricles and a thin corpus callosum. Metabolic screening was negative.

Patient 3 was referred at 5 years because he was suspected of having fragile X syndrome. He was born to healthy, non-consanguineous parents (25 year old mother) at 35.5 weeks of gestation. At birth, weight was 2700 g, height 46.5 cm, and head circumference 32 cm. During the neonatal period he had severe hypotonia. The boy was 14 months old when the first karyotype was studied. No dysmorphic features were mentioned except an open mouth. At 5 years, developmental delay was obvious and a second chromosome study was performed. At this age, weight was 21 kg and head circumference 48 cm (normal for age). The boy was able to say some words; he had marked instability without aggressive behaviour. Facial dysmorphic features were hypertelorism, deep set eyes, epicanthic folds, and convergent strabismus with slight ptosis. The ears were very large, sticking out, and posteriorly rotated. He had a narrow face with bitemporal narrowing (fig 1D, E). The mother had two normal children from another husband. She had 10 brothers and sisters out of which two brothers were not able to read or write. One was deaf and mentally retarded. One of the sisters had five children. Among them, one boy had learning disabilities. No information was available regarding the paternal family.

One important purpose of the present work was to develop a technological approach able to detect efficiently a cryptic chromosome deletion present at a very low frequency in large collections of patients, in the absence of DNA from the parents. We show here how Southern blotting and highly polymorphic minisatellites are one way to achieve this goal.

DNA samples from 567 patients were investigated. A deletion was suspected if the DNA showed only one band for at least two contiguous minisatellites. Such samples were then examined by comparing band intensities to distinguish between homo- or hemizygosity. Variations in DNA loading were estimated by using as controls two monomorphic minisatellites located on other chromosomes which were probed on the same membranes. Only three suspect samples were confirmed after this analysis. The three patients showed a single band at all five minisatellite loci and all three suspected deletions were subsequently confirmed by FISH. The deletion in patient 1 was of paternal origin and the deletion in patient 2 of maternal origin. The parents of the third case were unavailable for analysis. Phenotypic differences reflect the varying size of the deletion in good agreement with previous reports.^{6, 7} In particular, the deletion in patient 1 with severe mental retardation encompasses interval D1S243-D1S468 (table 1), which has been suggested to contain a gene for cognition, and is comparable to patients 21 to 2 in fig 3 of Wu *et al.*⁶

Taking into account a previous report,¹⁵ four cases of 1p36 deletion were identified among 666 patients, suggesting that 1p36 deletion is responsible for 0.5-0.7% of idiopathic mental retardation (according to the Poisson distribution, 96% confidence interval 0.15-1.2%). Assuming

that 40% of mental retardation is unexplained and that 2-3% of the population is affected by mental retardation, 1p36 deletion would affect between 1/10 000 and 1/100 000 newborns, in reasonably good agreement with earlier estimates (1/10 000⁶). All three cases identified here belong to the class of patients with moderate to severe impairment.

The precise definition of mental retardation phenotypes and the identification of candidate genes for mental retardation syndromes require that minimum deletion regions are defined as precisely as possible. The precision achieved depends to some extent upon the number of cases identified. Because the phenotype associated with a given syndrome is deduced directly from the minimum region defined at a given time, screening relying on phenotype only may miss patients with smaller deletions associated with a milder phenotype (as shown in Wu *et al*⁶ for the subclass of most distal 1pter deletions). However, large scale screening of patients for whom no diagnosis can be obtained raises many technical difficulties. Owing to the recent definition of the syndrome, and diffusion of this knowledge to clinicians, the majority of 1p36 deletions will now be detected by clinical examination. This implies that the relative frequency of, in this case, 1p36 deletions within unexplained mental retardation will decrease, and this will further enhance the need for low cost procedures in order to identify these rare, but potentially highly informative, minimally deleted patients.

Such procedures should not necessitate parental samples, at least for the first screen, since practice shows that this strongly diminishes the efficiency of any such approach and multiplies the costs. The recent availability of a telomeric probe panel has opened the way to relatively large scale screening (in the order of a few hundred subjects) for telomeric rearrangements.¹ However, the associated cost will probably limit the application of such procedures to patients with moderate to severe mental retardation representing approximately only 10% of all mentally retarded patients. In addition, in such a procedure, a single telomeric locus is usually assayed whereas interstitial deletions associated with the 1p36 syndrome have been reported.⁶

In this work, we present a first step towards an alternative approach enabling large scale screening at a lower cost. The procedure takes advantage of highly informative minisatellite probes and analyses DNA samples by Southern blotting. Here it was applied to 1p36 because, at present, it is one of the chromosome ends for which a sufficient number of appropriate minisatellites was available.¹² This limitation should be removed in the near future as larger parts of the human genome sequence are produced. Minisatellites cluster at a high density towards chromosome ends,¹¹ so it is expected that the necessary minisatellites will become available for any given human chromosome end (<http://minisatellites.u-psud.fr/>). Once the Southern blots have been produced, they are readily available for multiple hybridisation, owing to the fact that minisatellite probes usually provide very strong signals. Consequently, the data produced (DNA quantification) while screening one chromosome end is subsequently of use for other chromosome ends.

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A transmitted deletion of 2q13 to 2q14.1 causes no phenotypic abnormalities

EDITOR—An imbalance of genetic material, especially monosomy, will usually give rise to an abnormal phenotype. A few instances of proximal 2q deletions have been published, but previous cases (q12-q14,¹ q12-q14.2,² q14-q21^{3,4}) have been associated with clinical features such as mental retardation, facial dysmorphism, heart defects, and renal and digital anomalies.³ We ascertained an interstitial deletion of chromosome 2 at q13-q14.1 (fig 1) in a clinically normal G6, P2, SAB3 woman aged 38. She had been referred for chromosome analysis following three successive miscarriages at 8½, before 11, and at 7 weeks' gestation. Her current pregnancy was chromosomally normal at amniocentesis and continuing at 26 weeks. Testing of her two previous children was not being pursued at the time of writing. This deletion was subsequently found to have been transmitted by her G2, P2 mother who had no associated phenotype nor history of miscarriages. Cytogenetic analysis at the 600 band level failed to detect a subtle insertional translocation of the missing material elsewhere in the genome and the partners of both mother and daughter had normal karyotypes. Here we have attempted to define the extent of this deletion and thereby the degree of euchromatic loss using fluorescence in situ hybridisation (FISH).

Whole chromosome 2 paint (Cambio) corroborated our cytogenetic results in that no fluorescent signal was detected outside the two chromosomes 2 to account for the missing bands at 2q13-q14.1. To exclude an intrachromo-



Figure 1 Chromosome 2 with deletion indicated by bracket. G banded partial karyotype (left) and ideogram (right).

Table 1 Details of YAC for FISH

YAC name*	Database location*	Laboratory location†	STS No*	Distance from short arm telomere (cM)*	FISH result
636b10	q12-q13	q12-q13	D2S135	120	Normal
791f4	q13	q13	D2S1895	128	Partially deleted
817b4	q14	q14.1	D2S308	129	Deleted
786a12	q14	q14.1	D2S363	130	Deleted
679d2	q12-q14	q14.3	D2S110	135	Normal
821h9	q14	q14.3	D2S347	136	Normal
845f5	q12	q21	D2S1888	126	Normal
774a5	q35	q21	D2S112	146	Normal

*Data from the Max Planck Institute website.¹⁸

†Based on test and patient sample results.

somal insertion and to help identify the loci most proximal and distal to the deletion, a range of eight YACs (YAC Screening Centre, Milan) spanning proximal 2q were applied (table 1). At least five metaphases in either the proband or her mother were scored for a positive signal at each of these loci along with control probes at either the centromere (D2Z1) or distal to the deletion at 2q21 (845f5).

Results showed that both proximal 2q14 YACs 786a12 and 817b4 were deleted from the abnormal chromosome 2 (fig 2), while the distal 2q14 YACs 679d2 and 821h9 were retained. We conclude, therefore, that consistent with our findings from G banded analysis, the proximal region of 2q14 is absent. Hybridisation with the 2q13 YAC 791f4 produced a signal on both homologues, but one signal was frequently reduced in size. To confirm a partial deletion of this YAC, we carried out dual colour FISH in which YAC 791f4 was detected using texas red and one of the known deleted YACs with fluorescein (786a12). This showed that the reduced signal was consistently associated with the deleted homologue (fig 3). Semiquantitative FISH⁵ in a series of 10 cells showed that the signal from the partially deleted YAC was only 0.48 times (95% CI 0.32-0.72) that



Figure 2 FISH metaphase with YAC 786a12 (red signal) and the alphoid chromosome 2 centromere probe (green signals). Hybridisation was absent on the deleted chromosome, as indicated by the white arrow.