

# Further evidence for the involvement of human chromosome 6p24 in the aetiology of orofacial clefting

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## Abstract

**Chromosomal translocations affecting the 6p24 region have been associated with orofacial clefting. Here we present a female patient with cleft palate, severe growth retardation, developmental delay, frontal bossing, hypertelorism, antimongoloid slant, bilateral ptosis, flat nasal bridge, hypoplastic nasal alae, protruding upper lip, microretrognathia, bilateral, low set, and posteriorly rotated ears, bilateral microtia, narrow ear canals, short neck, and a karyotype of 46,XX,t(6;9)(p24;p23). The translocation chromosomes were analysed in detail by FISH and the 6p24 breakpoint was mapped within 50-500 kb of other breakpoints associated with orofacial clefting, in agreement with the assignment of such a locus in 6p24. The chromosome 9 translocation breakpoint was identified to be between D9S156 and D9S157 in 9p23-p22, a region implicated in the 9p deletion syndrome.**

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Human chromosome 6p has been implicated in the aetiology of orofacial clefting (OFC) by both linkage analysis<sup>1</sup> and by the coincidence of OFC with chromosomal abnormalities involving chromosome 6p.<sup>2,3</sup> In the most recent of these reports, we described two unrelated cases who presented with multiple congenital abnormalities including OFC, which were coincident with balanced translocations involving breakpoints within 6p24. These breakpoints were investigated by fluorescence in situ hybridisation (FISH) and were found to be within a region of the chromosome which was well characterised using YAC contig data, FISH mapping, and hybrid mapping data and were shown to be within 1 Mb of each other between markers D6S410 and D6S470.

In this paper we describe a further unrelated case with a balanced translocation between chromosomes 6 and 9 which again involves a breakpoint at 6p24. This subject, whose karyotype was interpreted as 46,XX,t(6;9)(p24;p23), has multiple congenital abnormalities including OFC. Since the 9p23 region has been implicated in the 9p deletion syndrome,<sup>4</sup> we have characterised both the 6p and 9p breakpoints by FISH using clones from contigs mapped to the two regions.

## Case report

The proband, a 7 month old girl, was the third child of a healthy, non-consanguineous couple aged 30 (mother) and 34 (father) years. The mother had a history of spontaneous abortion at 5 months' gestation, but both of the couple's previous children were healthy and phenotypically normal. The proband was born at term by normal delivery and with a birth weight of 3088 g; at 20 days she was diagnosed as having pyloric stenosis because of frequent vomiting after feeding and she subsequently underwent an operation. She was referred at 2 months of age because of poor suckling, poor weight gain, and a number of minor anomalies which were noted as follows: frontal bossing, hypertelorism, antimongoloid slant, bilateral ptosis, flat nasal bridge, hypoplastic nasal alae, protruding upper lip, microretrognathia, cleft palate, bilateral low set and posteriorly rotated ears, bilateral microtia, narrow ear canals, and short neck (fig 1). Radiographic examination showed a hypoplastic mandible. Her growth was significantly retarded and her mental development was severely delayed.

Chromosome analysis of the proband showed an apparently balanced reciprocal translocation between chromosomes 6 and 9, whereas the karyotype of each of her parents was normal. Therefore, her karyotype was 46,XX,t(6;9)(p24;p23) de novo.

## Materials and methods

### CELL CULTURE

Cells were obtained as an EBV transformed lymphoblast cell line. These were cultured at 37°C with 5% CO<sub>2</sub> in Dulbecco's Modified Eagles Medium containing 2 mol/l L-glutamine, 9% fetal calf serum, and 50 µg/ml gentamicin. Chromosome preparations were obtained by standard cytogenetic techniques. In brief, actively growing cells were incubated in the presence of thymidine at 280 µg/ml for approximately 18 hours; they were then washed in fresh medium and left to grow for five hours before treatment with colcemid, 75 mmol/l KCl, and fixation in 3:1 methanol acetic acid. Metaphase chromosomes were prepared on methanol washed slides and were then used for fluorescence in situ hybridisation (FISH) experiments as described below.

A lymphoblastoid cell line is available from both laboratories on request.

### FISH

Cosmid DNA was cultured, prepared, and purified by standard techniques. YAC clones were not isolated from endogenous yeast DNA

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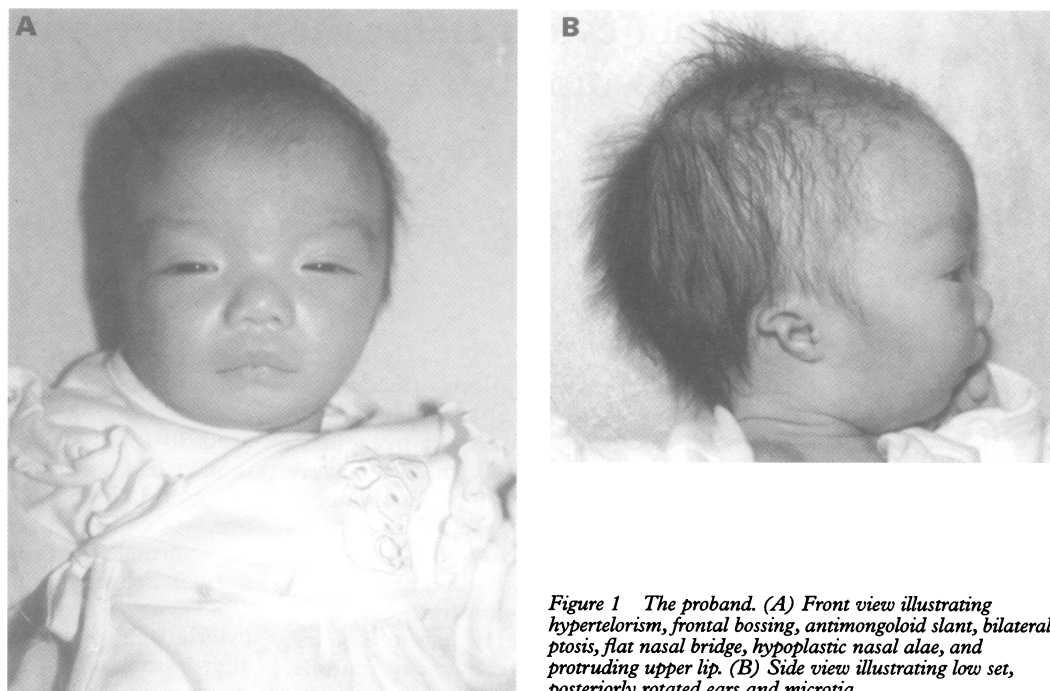


Figure 1 The proband. (A) Front view illustrating hypertelorism, frontal bossing, antimongoloid slant, bilateral ptosis, flat nasal bridge, hypoplastic nasal alae, and protruding upper lip. (B) Side view illustrating low set, posteriorly rotated ears and microtia.

before FISH; the total yeast DNA was prepared as described previously.<sup>5</sup> PAC DNA was prepared as recommended by the MRC HGMP Resource Centre. All clones were labelled with biotin-14-dATP or digoxigenin-11-dUTP by nick translation (Bio-Nick Labeling System or Nick Translation System respectively, BRL Life Technologies, USA).

In situ hybridisation was performed as described previously.<sup>6</sup> Briefly, probes for each slide were combined as required (50 ng of YAC per slide or 100 ng of cosmid or PAC per slide), dried down, and suspended in 50% formamide, 1% Tween-20, 20% dextran sulphate

along with salmon sperm DNA (100 × w/w) and Cot-1 DNA (50 × w/w). The probe mixes were then denatured by heating to 75°C for three minutes, prehybridised for 30 minutes, and applied to the slides. Hybridisation was carried out at 37°C for 16 hours. Signals from biotin labelled probes were developed using alternate layers of avidin-fluorescein-isothiocyanate (avidin-FITC) and biotinylated anti-avidin. Those from digoxigenin labelled probes were developed with a single layer of sheep antidigoxigenin conjugated to tetramethylrhodamineisothiocyanate (TRITC-antidigoxigenin) followed by one layer of donkey antisheep-TRITC. Slides were mounted in Vectashield antifading medium (Vector Laboratories, USA) containing 80 ng/ml 4',6-diamidino-2-phenylindole (DAPI) as counter-stain.

Signals were visualised under a Zeiss Axioplan microscope equipped with a cooled charge coupled device (CCD) camera (Photometrics, USA) and Smartcapture image analysis system (Vysis, UK). G banding was enhanced during image analysis. For the purposes of clone mapping, all clones were mapped to specific chromosome bands of normal male chromosomes by examination of a minimum of 10 well extended metaphases per clone. The quality of the banding was sufficient to allow localisation of clones to precise bands and sub-bands at the 550 band level. The mapped clones were then used to investigate the breakpoints involved in the case under investigation.

#### CLONE DETAILS

All chromosome 6p specific YAC clones and cosmid 6K23 had been previously characterised and mapped by FISH.<sup>2,5,7</sup> The cosmid clones B2.2, B10.10, F1.6, A9.5, E11.2, G10.5, B11.7, C8.7, and C2.3 had been isolated from the ICRF chromosome 6 specific cosmid library using YAC 886c1 and mapped

Table 1 Results of FISH on the der(6) and der(9)

Clone name	Chromosomal location by FISH	Signal
der(6)		
6K23	6p25.3	-
947_d_4	6p25	-
B19		-
887_h_3	6p24.3/p25	-
808_a_10	6p24.3	-
826_a_12	6p24.3	-
886_c_1	6p24.3	+/-
938_d_8	6p24.2	+/-
B2.2	6p24.2	-
B10.10	"	-
F1.6	"	-
A9.5	"	-
E11.2	"	-
G10.5	"	-
B11.7	"	+
C8.7	"	+
C2.3	"	+
242F16	"	+
933_c_3	6p24.2	+
844_h_3	6p23/24	+
der(9)		
853_f_4		-
783_h_10		-
912_e_9		-
806_f_7		-
952_g_4		-
937_c_8		+
776_f_2		+
795_b_10		+
915_g_3		+
929_g_12		+

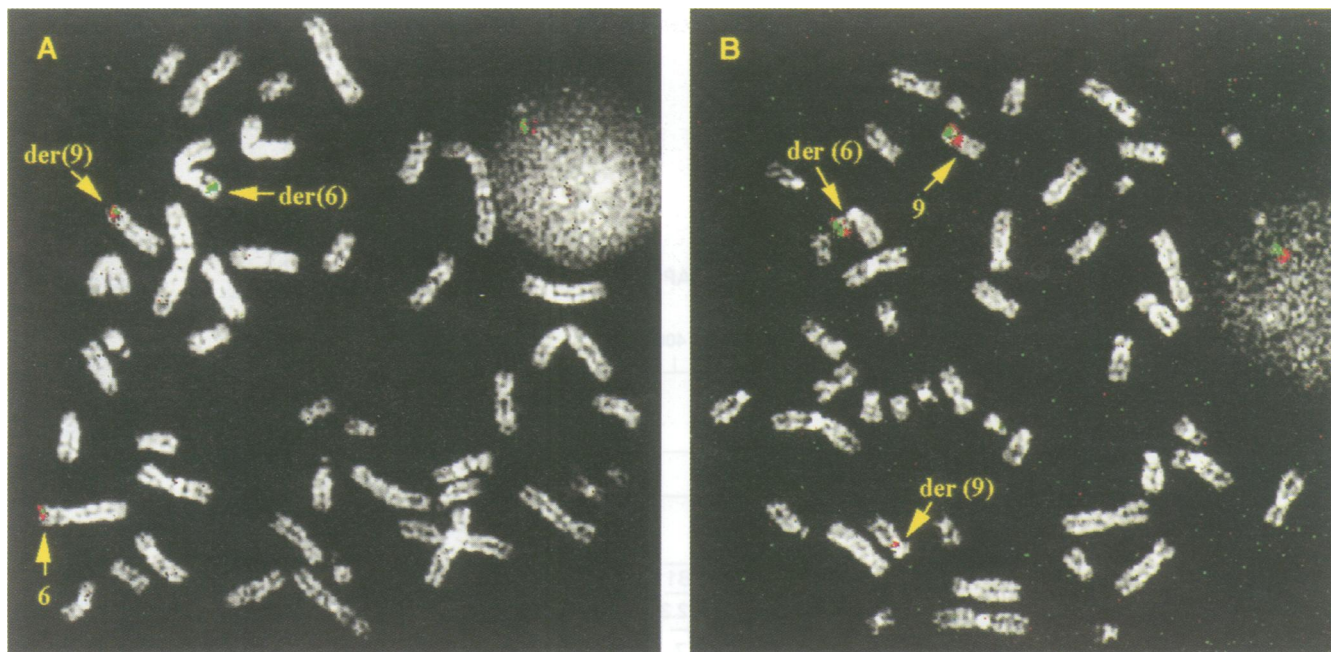


Figure 2 FISH results on the patient's chromosomes. (A) FISH using YACs 938d8 (green) and 808a10 (red). 938d8 is present on chromosome 6, der(6), and der(9), whereas 808a10 is present only on 6 and der(9). Therefore, 938d8 crosses the translocation breakpoint and 808a10 lies distal to the breakpoint. (B) FISH using YACs 912e9 (red) and 783h10 (green). Both YACs are located on chromosomes 9 and der(6) indicating that they are located distal to the chromosome 9 breakpoint. A marker probe (Imagenetics, red) was used for the unambiguous identification of chromosome 9.

within 6p24.2 in that order from the telomere to the centromere (Stephens *et al*, manuscript in preparation). In particular, the cosmids B11.7, C8.7, and C2.3 contain the AP-2 gene. The PAC clone 242F16 (UK-HGMP-Resource Centre) was isolated using the marker D6S470 (E Bentley, unpublished data). The YAC clones 853\_f\_4, 783\_h\_10, 912\_e\_9, 806\_f\_7, 953\_g\_4, 937\_c\_8, 776\_f\_2, 795\_b\_10, and 929\_g\_12 were mapped previously to chromosome 9p by the Whitehead Institute.<sup>10</sup> All clones were reported to map in the 9p23-p22 region and contain the following genetic markers: 853\_f\_4-D9S289 (17 cM), 783\_h\_10-D9S267 (25 cM), 806\_f\_7-D9S1869 (26 cM) and D9S274 (28 cM), 912\_e\_9-D9S274 (28 cM), 953\_g\_4-D9S156 (30 cM), 937\_c\_8-D9S157 (32 cM), 776\_f\_2 and 795\_b\_10-D9S162 (33 cM), 929\_g\_12-D9S171 (42 cM).

## Results

### MAPPING OF THE TRANSLOCATION BREAKPOINT

#### ON 6p

The translocation breakpoint in 6p24 was determined by using a selection of probes covering 6p23-pter and FISH. Thus all clones mapping to 6p24.3 and 6p25 were present on the derivative 9 chromosome, while probes located within 6p24.2 to 6p23 were present on the derivative 6 (table 1, fig 2A). Signals from the overlapping YACs 886c1 and 938d8 were found on both derivative chromosomes indicating that the breakpoint is located within the segment of overlap of these clones (table 1, fig 2A, fig 3).

Further fine localisation was performed by using bacterial clones that had previously been mapped along YAC clone 886c1 (Stephens *et al*, manuscript in preparation). Eight cosmids and one PAC clone containing the marker D6S470 were used (fig 3). The 1600 kb long

YAC clone 886c1 can be subdivided into two intervals: the distal 800 kb that contains YAC clone 808a10 and the two translocation breakpoints reported in Davies *et al*<sup>2</sup> and the proximal 800 kb interval that overlaps with the YAC 938d8 (fig 3). This latter region contains the markers D6S470 and AP-2. All cosmids derived from the distal 800 kb interval were present on the derivative 9 chromosome, while the probes covering the proximal interval were found on the derivative 6 (table 1, fig 3). The breakpoint was localised between cosmids G10.5 and PAC 242F16, centromeric of the two previously reported breakpoints at an approximate distance of 50-500 kb from the nearest breakpoint. Therefore all three breakpoints are contained within an 800 kb interval on YAC 886c1 (fig 3).

### MAPPING OF THE TRANSLOCATION BREAKPOINT

#### ON 9p

Since the chromosome 9 breakpoint was determined cytogenetically to lie at 9p23, YAC clones reported to contain markers localised in the 9p23-p22 interval were used to confirm the position of the breakpoint and investigate its position compared to 9p23 deletion breakpoints.<sup>8</sup> Ten clones were used containing markers mapped at a genetic distance of 17 cM to 42 cM from the telomere of 9p (table 1, fig 2B, fig 3). Of these, five clones containing markers at 17 cM (D9S286) to 30 cM (D9S156) were present on the derivative chromosome 6, while the YAC clones containing markers at 32 cM (D9S157) to 42 cM (D9S171) were located on the derivative 9. These data assign the breakpoint to a position between 30 and 32 cM from the telomere on chromosome 9p, within the 9p23-p22 region (fig 3).

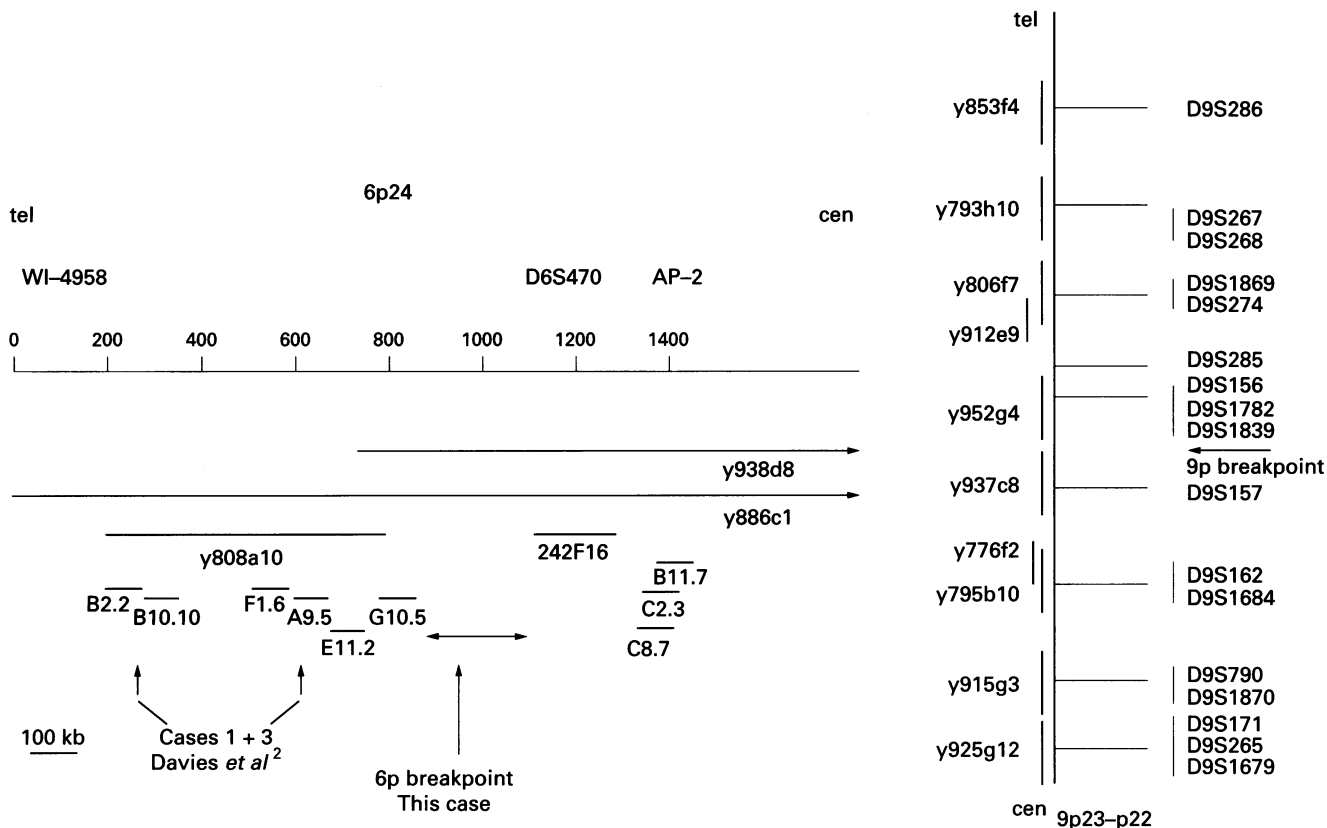


Figure 3 Map of the 6p24 region (left) showing markers and distances (above), YAC, PAC, and cosmid clones (below). The translocation breakpoints are indicated by arrows. To the right a schematic diagram of the 9p23-p22 region is shown. YAC clones are represented by vertical bars (left) and markers are shown to the right. The arrow indicates the position of the breakpoint.

### Discussion

The case described here presented with multiple craniofacial anomalies, most of which are consistent with the 6p deletion syndrome, such as microretrognathia, frontal bossing, hypertelorism, flat, broad nasal bridge, low set ears, and developmental delay.<sup>10, 11</sup> Cleft palate is consistent with the phenotype of other 6p24 translocation cases described.<sup>2, 3</sup> However, congenital malformations and developmental delay are associated with orofacial clefting and in particular with 22% of non-syndromic isolated cleft palate in a recent study.<sup>12</sup> Therefore it is possible that the largest part of the phenotypic spectrum of this case is caused by one locus affected by the translocation.

The detailed investigation by FISH using well characterised probes allowed the direct comparison with other cases; in particular the use of YACs containing genetic markers for chromosome 9 has enabled a direct comparison to cases presented recently.<sup>8</sup>

The chromosome 6 breakpoint is located in a region 50-500 kb proximal to the breakpoints of cases 1 and 3 described in Davies *et al*<sup>2</sup> and at least 200 kb distal to the AP-2 gene. No deletions at cosmid clone level were observed in any of the three cases. Therefore, either a single gene spanning a large interval is disrupted by the translocations or its expression is altered because of position effects, as for example in Rieger syndrome translocations and RIEG.<sup>13</sup> It is also possible that position effects involve the nearby AP-2 gene. Interestingly, 4% of the otherwise healthy heterozygous AP-2 knock out mice present with

micrognathia,<sup>14</sup> which is part of the patient's phenotype.

The 9p breakpoint maps in the region between D9S156 and D9S157 in 9p23-p22, which is part of a segment with a high incidence of breakpoints. Although the patient does not have the typical characteristics of the 9p deletion syndrome, such as trigonocephaly, midface hypoplasia, upward slanting palpebral fissures, long philtrum, and mental retardation, she does share the latter and other unspecific 9p deletion findings like short nasal alae, narrow ear canals, and low set, posteriorly rotated ears. Therefore it is conceivable that genes (or a gene) on 9p are disrupted by the translocation and contribute(s) to the complexity of the phenotype.

An additional interesting aspect of the 6;9 translocation is that both breakpoints are located within regions that are frequently involved in translocations or deletions of the respective chromosome.<sup>2, 8, 11</sup> The presence of repeat sequences has been implicated in such phenomena as shown in chromosomal deletions causing Prader-Willi or Angelman syndrome.<sup>15</sup> The molecular characterisation of the breakpoints described here may lead to an understanding of the mechanism involved in the translocation, as well as the identification of genes playing a role in craniofacial development.

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- 1 Eiberg H, Bixler D, Nielsen L, Conneally PM, Mohr J. Suggestion of linkage of a major locus for nonsyndromic orofacial cleft with F13A and tentative assignment to chromosome 6. *Clin Genet* 1987;32:129-32.
- 2 Davies A, Stephens R, Olavesen M, et al. Evidence of a locus for orofacial clefting on human chromosome 6p24 and STS content map of the region. *Hum Mol Genet* 1995;4:121-8.
- 3 Donnai D, Heather L, Sinclair P, Thakker Y, Scambler P, Dixon M. Association of autosomal dominant cleft lip and palate and translocation 6p23;9q22.3. *Clin Dysmorphol* 1992;1:89-97.
- 4 Huret JL, Leonard C, Forestier B, Rethore MO, Lejeune J. Eleven new cases of del(9p) and features from 80 cases. *J Med Genet* 1988;25:741-9.
- 5 Olavesen MG, Davies AF, Broxholme SJ, et al. An integrated map of human chromosome 6p23. *Genome Res* 1995;5:342-58.
- 6 Adinolfi M, Davies AF. *Non-isotopic in situ hybridisation. Applications to clinical diagnosis and molecular genetics*. Austin, TX: R G Landes Co, 1994.
- 7 Davies AF, Olavesen MG, Stephens RJ, et al. A detailed investigation of two cases exhibiting characteristics of the 6p deletion syndrome. *Hum Genet* 1996;98:454-9.
- 8 Schwartz S, Crowe C, Conroy J, Haren J, Micale M, Becker L. Chromosome breakage hot spots in 9p and delineation of the critical region for the 9p deletion syndrome. 1996. [scw9, www.gne.ucl.ac.uk/chr9/scw96/abstract.html](http://www.gne.ucl.ac.uk/chr9/scw96/abstract.html)
- 9 Whitehead Institute for Genome Research human physical mapping data release. 12/11/1997: [http://www-genome.wi.mit.edu/cgi-bin/contig/phys\\_map](http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map)
- 10 Palmer C, Bader P, Slovak M, Comings D, Pettenati M. Partial deletion of chromosome 6p: delineation of the syndrome. *Am J Med Genet* 1991;39:155-60.
- 11 Ragoussis J, Davies AF, Mirza G, et al. Detailed characterization of six cases with 6p deletions by FISH: delineation of the syndrome. *Am J Hum Genet* 1997;61:A157.
- 12 Milerad J, Larson O, Hagberg C, Ideberg M. Associated malformations in infants with cleft lip and palate: a prospective, population based study. *Pediatrics* 1997;100:180-6.
- 13 Flomen RH, Vatcheva R, Gorman PA, et al. Construction and analysis of a sequence-ready map in 4q25: Rieger syndrome can be caused by haploinsufficiency of RIEG, but also by chromosome breaks approximately 90 kb upstream of this gene. *Genomics* 1998;49:409-13.
- 14 Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* 1996;381:235-8.
- 15 Christian SL, Martin SA, Fantes J, Bhatt NK, Huang B, Ledbetter DH. Identification of a large duplicated gene cluster region at the common deletion breakpoints of Prader-Willi and Angelman syndromes. *Am J Hum Genet* 1997;61:A24.