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A Locus for Isolated Cleft Palate, Located on Human Chromosome 2q32

Carole M. Brewer,[1] Jack P. Leek,[2] Andrew J. Green,[3] Susan Holloway,[1] David T. Bonthron,[1] Alexander F. Markham,[2] and David R. FitzPatrick[1]

[1]Human Genetics Unit, University of Edinburgh, and Department of Clinical Genetics, Western General Hospital, Edinburgh; [2]Molecular Medicine Unit, St. James's University Hospital, University of Leeds, Leeds; and [3]Department of Medical Genetics, Addenbrooke's Hospital, University of Cambridge, Cambridge

Summary

We present evidence for the existence of a novel chromosome 2q32 locus involved in the pathogenesis of isolated cleft palate. We have studied two unrelated patients with strikingly similar clinical features, in whom there are apparently balanced, de novo cytogenetic rearrangements involving the same region of chromosome 2q. Both children have cleft palate, facial dysmorphism, and mild learning disability. Their karyotypes were originally reported as 46, XX, t(2;7)(q33;p21) and 46, XX, t(2;11)(q33;p14). However, our molecular cytogenetic analyses localize both translocation breakpoints to a small region between markers D2S311 and D2S116. This suggests that the true location of these breakpoints is 2q32 rather than 2q33. To obtain independent support for the existence of a cleft-palate locus in 2q32, we performed a detailed statistical analysis for all cases in the human cytogenetics database of nonmosaic, single, contiguous autosomal deletions associated with orofacial clefting. This revealed 2q32 to be one of only three chromosomal regions in which haploinsufficiency is significantly associated with isolated cleft palate. In combination, our data provide strong evidence for the location at 2q32 of a gene that is critical to the development of the secondary palate. The close proximity of these two translocation breakpoints should also allow rapid progress toward the positional cloning of this cleft-palate gene.

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Address for correspondence and reprints: Dr. David T. Bonthron, Human Genetics Unit, University of Edinburgh, Molecular Medicine Centre, Western General Hospital, Edinburgh, UK, EH4 2XU. E-mail: D.Bonthron@ed.ac.uk

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Introduction

Isolated cleft palate (CPO; MIM 119540) is a common human malformation, with a total birth incidence of 1 in 1,250 in western Scotland (FitzPatrick et al. 1994). Both significant familial clustering (Shields et al. 1981; Carter et al. 1982; Christensen and Fogh-Andersen 1993) and twin studies (Shields et al. 1979) have suggested that there is a major genetic component in the etiology of CPO. These studies and others (Fogh-Andersen 1942) have also shown that CPO and cleft lip, with or without cleft palate (CL[P]), are genetically distinct subgroups of orofacial clefting. CPO is a common feature of chromosomal abnormalities, affecting ~15% of all cases of simple autosomal aneuploidy (Schinzel 1994; Brewer et al. 1998) and is associated with more than 370 different malformation syndromes (Winter and Baraitser 1996). However, in nonsyndromic CPO, relative risk ratio analyses have indicated that there may be a relatively small number of interacting causative loci (FitzPatrick and Farrell 1993; Christensen and Mitchell 1996). As yet, no disease-causing mutations in nonsyndromic CPO have been identified. In this study, we report clinical, cytogenetic, molecular, and statistical evidence for the existence of a previously unrecognized locus for CPO, located at 2q32.

Subjects and Methods

Case 1

This patient, the fourth child of healthy, nonconsanguineous parents, was delivered at 38 weeks' gestation, weighing 2.95 kg. She had a cleft palate at birth. Her delayed motor development was apparent at age 4 mo, and she did not walk until age 2 years. Particular problems were noted with the acquisition of language skills. She underwent repair of her cleft palate at age 18 mo and required pharyngoplasty at age 11 years. Her hearing is normal. She has a prominent nasal bridge with underhanging columella; a small mouth with distinctive upper lip; and long, slender fingers (fig. 1*A*, *B*). Her growth has been satisfactory, and her height has always been in or above the 50th percentile. Her weight was

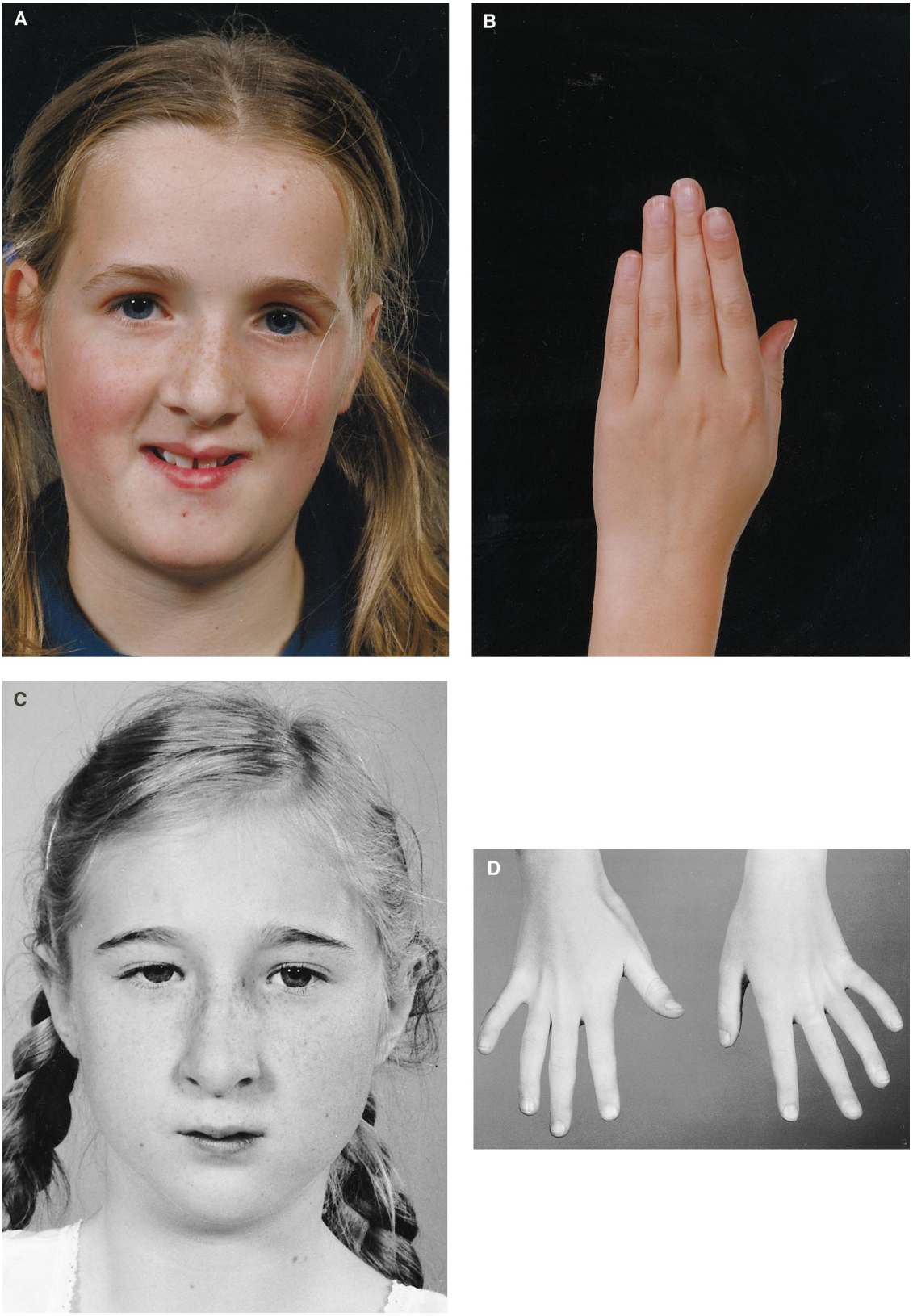

Figure 1 *A,* Patient 1: Aged 13 years, with a narrow nasal root, a prominent nasal bridge, and a small mouth with distinctive upper lip. *B,* Patient 1: Hands with long, slender fingers. *C,* Patient 2: Aged 11 years, with a prominent nasal bridge, a small mouth with distinctive upper lip, and mild micrognathia. *D,* Patient 2: Hands with long, slender fingers and fifth-finger clinodactyly.

below the 10th percentile until age 5 years but at age 10 years is in the 75th percentile. Her head circumference is in the 50th percentile. She has moderate learning disability. Blood chromosome analysis revealed an apparently balanced reciprocal translocation with the karyotype 46, XX, t(2;7)(q33;p21) (fig. 2*A*). Parental karyotypes were normal.

Case 2

This patient, delivered at term after an uneventful pregnancy, had, at birth, a cleft palate and minor facial dysmorphism. In addition to repair of her palate, she has required surgical correction of a convergent squint. On examination, at age 8 years, she has fair hair and skin; a long, narrow face with apparent hypotelorism; a prominent nasal bridge and slightly underhanging columella; a small mouth with distinctive upper lip (fig. 1*C*); mild micrognathia; and long, slender fingers with fifth-finger clinodactyly (fig. 1*D*). She has abnormal dermatoglyphics, with a reduced ridge count. She is of slender build, with height between the 75th and 90th percentiles and weight in the 10th percentile. She has mild global developmental delay, particularly in language skills, and is 1 year behind her peers in a mainstream school. Chromosome analysis revealed an apparently balanced reciprocal translocation with the karyotype 46, XX, t(2;11)(q33;p14) (fig. 2*B*). Parental karyotypes were normal.

In both cases, a microdeletion of the velocardiofacial syndrome (VCFS; MIM 192430) region of 22q11.22 was excluded both by FISH and by showing heterozygosity for at least three of the microsatellite markers—D22S420, D22S1638, D22S941, D22S1648, D22S944, and D22S264—that map to this region. For any apparently homozygous marker within the region, the parental haplotypes were also consistent with biparental contributions to both girls (data not shown). Clinical and cytogenetic studies were approved by the appropriate local ethics committees, and informed consent was obtained from both subjects' parents.

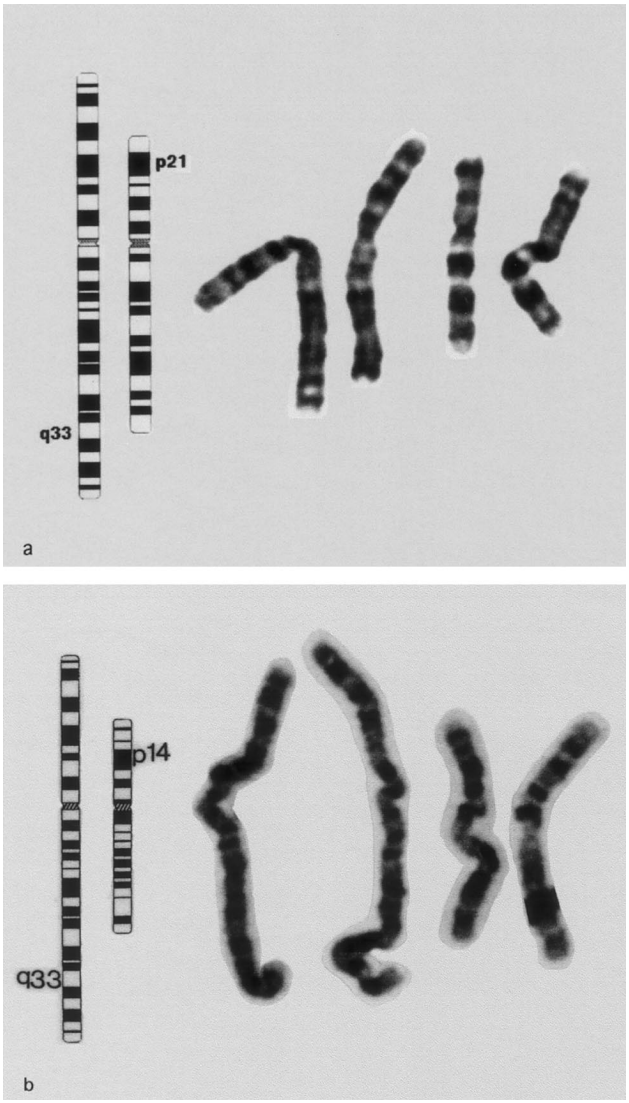

Figure 2 *A*, G-banded chromosome preparation from patient 1, showing apparently balanced reciprocal translocation; 46, XX, t(2;7)(q33;p21). *B*, G-banded chromosome preparation from patient 2, showing apparently balanced reciprocal translocation; 46, XX, t(2;11)(q33;p14).

Isolation of Genomic Probes from Chromosome 2q32

A series of oligonucleotide primers for loci tentatively mapped to 2q32 (Dib et al. 1996) were used to screen the Imperial Chemical Industries (ICI) yeast artificial chromosome (YAC) library (Anand et al. 1990) by PCR. The markers chosen, and their corresponding YAC clones, are presented in table 1. PCR reactions (30 μl) contained 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.25 μM forward and reverse primer, 1 U of *Taq* DNA polymerase (Promega), 50 ng of YAC-pool DNA, and 1 × reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100). Mg^{2+} concentrations were optimized between 0.5–2.5 mM. Reactions were cycled 30 times at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. YAC-clone DNA was isolated as described elsewhere (Riley et al. 1990).

Our initial working map across the 2q breakpoint region (Dib et al. 1996) and a modified physical map, established on the basis of the consensus map of Collins et al. (1996), data from our FISH studies, and a recent CEPH YAC contig (Hadano et al. 1999), are presented in figure 3. In addition to the anonymous 2q32 markers, we isolated YAC clones containing a number of genes known to be located in this region but which had not

Table 1

YAC Clones Used for FISH Analysis of CPO translocations

Marker and YAC	Chimeric by FISH	Position[a]
D2S311:		
17GD1	+	Proximal
31CH5	−	Proximal
33AC9	−	Proximal
D2S348:		
19ID10	ND[b]	Not applicable
21GA12	ND	Not applicable
23EG11	ND	Not applicable
32EB9	ND	Not applicable
D2S116:		
6HA11	−	Distal
13BE7	ND	Not applicable
14DE4	ND	Not applicable
16IB4	+	Distal
28DE5	ND	Not applicable
D2S309:		
4EC12	+	Distal
31DH5	+	Distal
D2S72:		
26IF5	−	Distal
D2S105:		
26IF5	−	Distal
CTLA4:		
26IF5	−	Distal
8IH5	ND	Not applicable
13HC12	ND	Not applicable
20AG2	ND	Not applicable
22HB6	ND	Not applicable
D2S307:		
26IF5	−	Distal
22HB6	ND	Not applicable
6BC7	ND	Not applicable
D2S1384:		
26IF5	−	Distal
22HB6	ND	Not applicable
10GF2	ND	Not applicable
WI5293:		
26IF5	−	Distal
8BG7	ND	Not applicable
14HA2	−	Distal
24GF8	ND	Not applicable
D2S2189:		
8BG7	ND	Not applicable
14HA2	−	Distal
23CE7	ND	Not applicable
24GF8	ND	Not applicable
D2S1271:		
15BA12	ND	Not applicable
22HC8	ND	Not applicable
FN1:		
7AH3	+	Distal
11GH11	ND	Not applicable
31GG9	−	Distal
37HB8	ND	Not applicable
IGFBP5:		
21EC3	−	Distal
22DB10	ND	Not applicable
IGFBP2:		
7FA11	ND	Not applicable
8HF12	ND	Not applicable
21EC3	−	Distal
22DB10	ND	Not applicable
IHH:		
35EF10	−	Distal

[a] A plus sign (+) denotes a chimeric YAC; a minus sign (−) indicates a nonchimeric YAC; and "ND" denotes that FISH was not done.

[b] Relative to translocation breakpoints.

been finely mapped. These were selected for their possible involvement in the etiology of cleft palate (table 1).

FISH

Metaphase chromosome spreads were prepared from peripheral blood lymphocytes as described elsewhere (Gosden 1990). Whole-chromosome paints for chromosomes 2, 7, and 11, and the centromere repeat probes D2Z1, D7Z1, and D11Z1, directly labeled with fluorescein or Texas red, were obtained from Appligene/Oncor. To generate a third color, fluorescein- and Texas red–labelled D2Z1 were mixed in a 1:1 ratio prior to use. YAC DNAs were labeled with digoxigenin-11-dUTP (Boehringer-Mannheim) by nick translation (Rigby et al. 1977) by means of a kit optimized for use with large-fragment probes (Appligene/Oncor). The reaction was allowed to proceed until fragment sizes averaged 300–600 bp. The probe was then ethanol precipitated along with a 20-fold excess of human Cot-1 DNA (Gibco BRL) and redissolved in hybridization buffer containing 10% dextran sulphate, 50% formamide, 2 × SSC, and 50 mM phosphate buffer, pH 7.0.

YAC probes were denatured at 75°C for 10 min and then preannealed at 37°C for 1 h. Chromosome paints and centromere probes were denatured at 75°C for 5 min, and chromosomal DNA was denatured at 70°C for 3 min, followed by quenching and dehydration in a cold ethanol dilution series (70%, 90%, 100%). Hybridization was done under a sealed 22 × 22 mm coverslip at 37°C for 16 h. Posthybridization washing was in 1 × SSC at 70°C for 5 min. YAC-probe hybridization sites were labeled with Fab fragments of sheep antidigoxigenin (Boehringer-Mannheim) and FITC-conjugated rabbit antisheep IgG (Sigma). Slides were mounted in antifade solution containing 0.1 μg/ml 4,6 diamidino-2-phenylindole (DAPI) (Appligene/Oncor).

Images were captured by use of a Zeiss Axioskop fluorescence microscope, fitted with a cooled CCD camera. Analysis was done on a Vysis-Quips Genetic Workstation (Vysis) and map positions were determined by observation of the DAPI banding pattern by means of Smart Capture VP version 1.3.1 software. YAC probes that proved to be chimeric by FISH analysis are recorded in table 1.

Statistical Analysis of Chromosomal Deletions

The statistical reanalysis of the CPO cases in the human cytogenetics database (HCDB) was done as described elsewhere (Brewer et al. 1998). In brief, the number of CPO-associated deletions of each band was compared with the expected number, calculated from the distribution of all band deletions in HCDB. The number of deletions of any band was taken to follow a Poisson

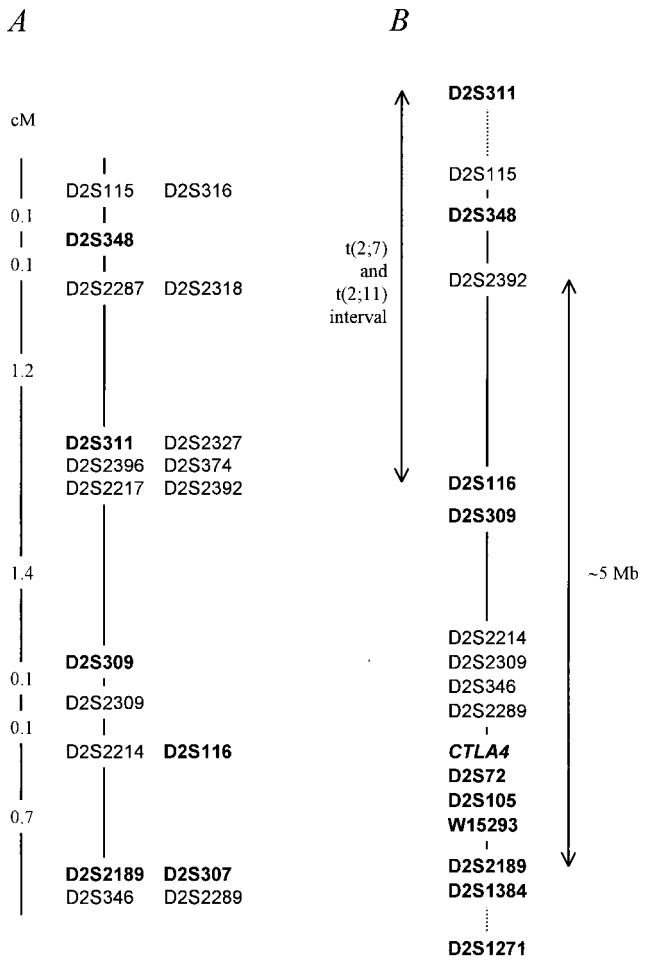

Figure 3 *A,* Original genetic map of the 2q32 region (Dib et al. 1996). *B,* Physical map of the region on the basis of the work of Hadano et al. (1999), with inclusion of data from LDB (Collins et al. 1996) and the present study. Discrepancies in marker order have been discussed (Hadano et al. 1999). Markers for which YACs were identified in the present study are shown in boldface type.

distribution, since this number is usually small. Confidence limits for the observed number of deletions and the significance of any deviation from the expected number were calculated as described by Vasarhelyi and Friedman (1989).

Results

Patient Phenotypes

Initial clues to the existence of a CPO locus on 2q32–33 came through the ascertainment of a patient (case 2) with a de novo balanced reciprocal translocation, t(2;11)(q33;p14). By further inquiry, we were then able to identify a second patient (case 1), again with CPO and a de novo translocation, t(2;7)(q33;p21), involving the same cytogenetic band on chromosome 2. Both patients were personally examined by C.M.B., and their clinical appearances are strikingly similar (fig. 1).

FISH Analysis

Initial chromosome painting studies confirmed that the sizes of the translocated 2q fragments were approximately the same in both patients (fig. 4*A, B*). To establish whether the breakpoints in cases 1 and 2 lie within the same region of chromosome 2q, an extensive single-locus FISH analysis was then conducted by means of a collection of YACs, containing markers mapping within the 2q32–33 region (fig. 3). The results of this FISH study are summarized in table 1. YACs containing the candidate genes fibronectin (*FN1*; MIM 135600), insulin-like growth factor binding proteins *IGFBP5* (MIM 146734) and *IGFBP2* (MIM 146731), and Indian hedgehog (*IHH*; MIM 600726) were all found to map distal (telomeric) to both chromosome 2 breakpoints (fig. 4*C–F*).

Anonymous markers flanking both patients' 2q breakpoints were next identified by FISH. The signals generated by YACs containing D2S311 are present on both the normal and derivative copies of chromosome 2, in both cases 1 and 2 (fig. 4*G, H*). Thus D2S311 is proximal (centromeric) to both breakpoints; this is made unambiguous through simultaneous hybridization to the centromeric probes D2Z1 and either D7Z1 or D11Z1. In contrast, FISH with YAC 14HA2 (containing D2S2189) gave signals lying distal to the breakpoint in both individuals (data not shown). This suggested that both breakpoints lie within a common region (D2S311–D2S2189) of some 6 Mb of 2q32. To reduce this interval further, the following YACs were analyzed: 26IF5 (containing D2S1384/D2S307/*CTLA4*/D2S105/D2S72); 4EC12 and 31DH5 (both chimeric, but containing D2S309); and 6HA11 (containing D2S116;fig. 4*I, J*). All were found to map distal to the 2q32 breakpoint in both patients. In this way, by systematic FISH analysis, the breakpoint region in both these patients with cleft palate has been localized to an interval (D2S311–D2S116) that may be as small as 2.5 Mb, according to current maps (Collins et al. 1996).

Chromosome Deletion Map for CPO

By analyzing all cases of single, contiguous, nonmosaic autosomal deletions in the HCDB (Schinzel 1994), we previously identified three chromosomal regions—2q32, 4p16–13, and 4q31–35—where monosomy is nonrandomly associated with CPO (Brewer et al. 1998). However, HCDB searches alone do not differentiate cleft palate in the context of CL(P) from CPO cases. As these are etiologically distinct subgroups of

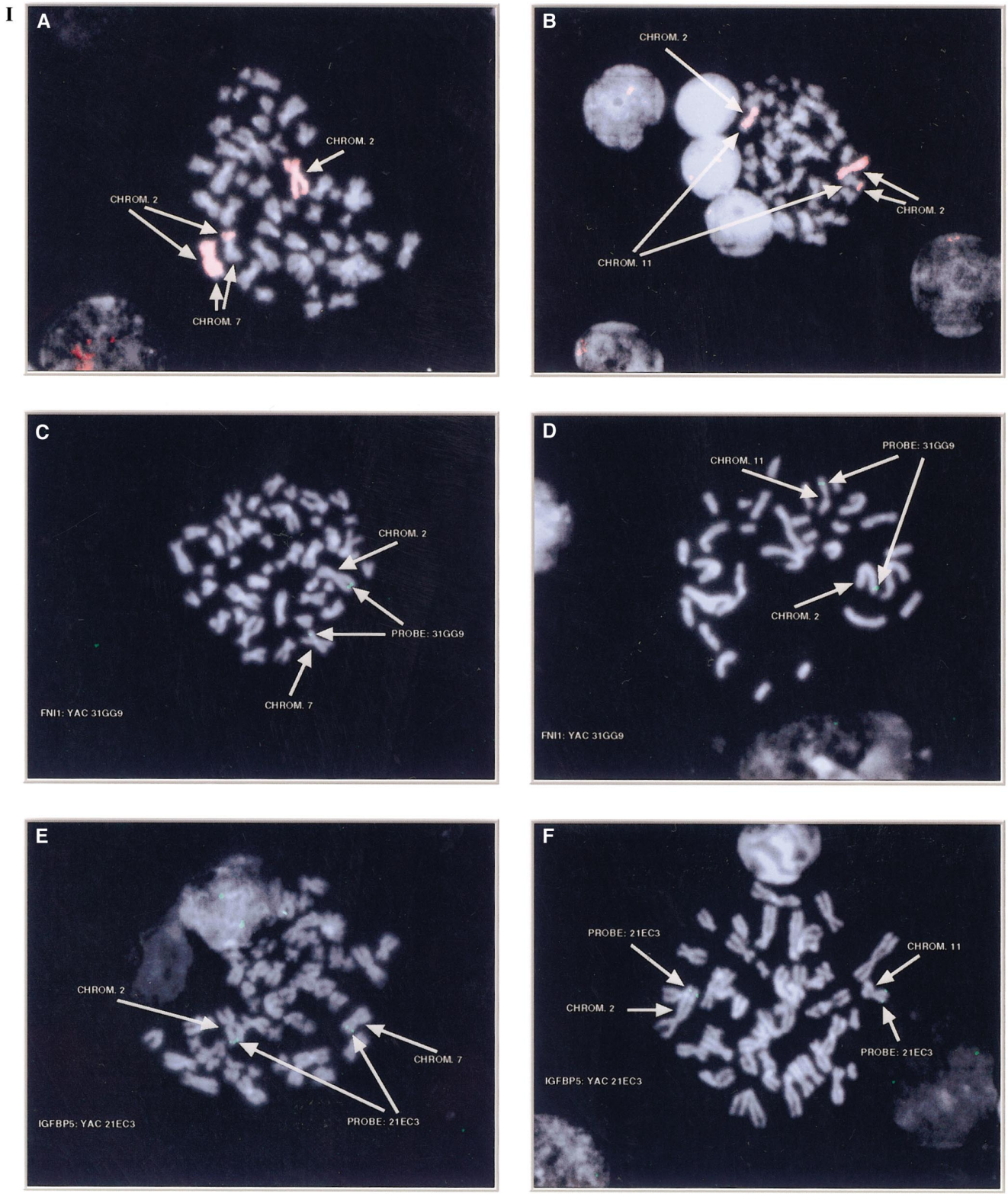

Figure 4 FISH analysis of patient chromosomes. *A*, Case 1 and *B*, Case 2: metaphase chromosomes analyzed with a chromosome-2 paint. *C*, Case 1 and *D*, Case 2: analyzed by single-locus FISH with a YAC (31GG9) containing *FN1*. *E*, Case 1 and *F*, Case 2: chromosomes analyzed by single-locus FISH with a YAC (21EC3) containing the *IGFBP5* gene. *G*, Case 1 and *H*, Case 2: analyzed with a YAC containing D2S311 (31CH5, labeled green) and the centromere probes D2Z1 (yellow) and either D7Z1 or D11Z1 (red). *I*, Case 1 and *J*, Case 2: analyzed with a YAC probe (6HA11) containing D2S116 (green signal). Centromere probes are as described earlier.

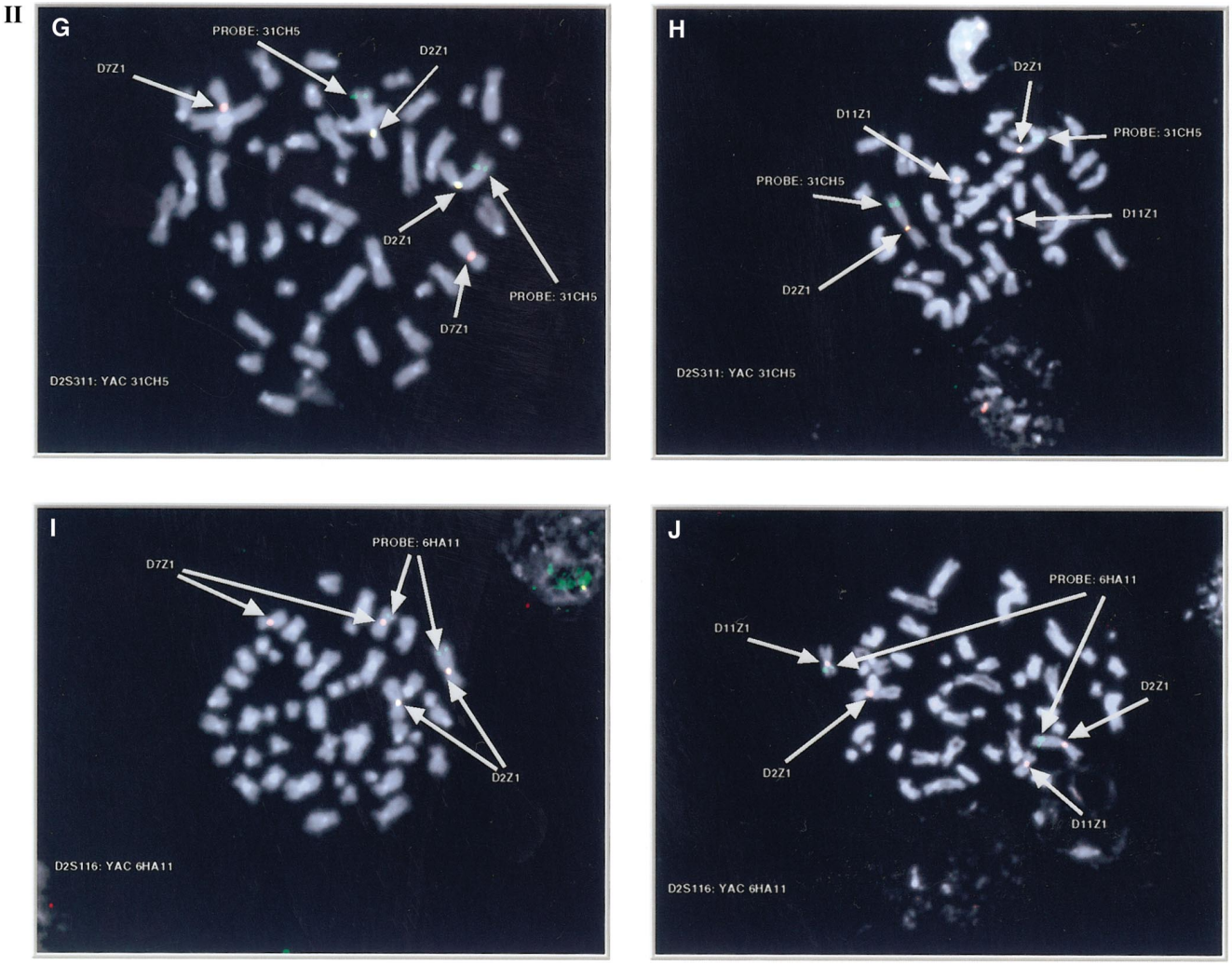

Figure 4 (*continued*)

orofacial clefting, they are expected to have different causative genetic loci. It was possible to address this problem because, for each band, the total number of deletions and the number of cases of cleft lip and of cleft palate were known. After subtracting the number of cases associated with cleft lip from the number of cases associated with cleft palate, any remaining cases of cleft palate associated with deletion of that autosomal band must have CPO. There were 269 cases of simple autosomal deletion associated with cleft palate and 95 cases associated with cleft lip. Of the cleft palate cases, 65% could be assigned unambiguously as CPO. When the band distribution of cleft-palate deletions was examined, 782 deleted bands were associated with cleft palate, and among these bands there were 294 cleft-lip associations, leaving 62% of band deletions that were unequivocally associated with CPO. The results of both the original analysis (Brewer et al. 1998) and this reanalysis of CPO deletion cases are shown in the Appendix. Each of the three cleft palate–associated chromosomal regions identified elsewhere was confirmed as a CPO-associated region in the new analysis. No new CPO-associated regions were identified.

Discussion

On the basis of two independent lines of evidence, we suggest that a previously unrecognized locus causing cleft palate resides in chromosome region 2q32. This suggestion relies on the integration of clinical, cytogenetic, molecular, and statistical data. Our studies were initiated by the observation of two unrelated children with strikingly similar clinical features, each having a de novo cytogenetic rearrangement apparently involving the same band on chromosome 2q. Both girls have cleft palate, mild learning difficulties, and a strikingly similar

facial appearance (fig. 1). Although their facial dysmorphisms are subtle, these and other clinical features are reminiscent of those seen in VCFS. It would appear, however, that these girls do not have VCFS, as neither of them has a cardiac malformation, nor the microdeletion of 22q11.22 seen in most cases of VCFS (Scambler et al. 1992). The existence of non-22q–deleted phenocopies of VCFS is well recognized (Daw et al. 1996), and it is possible that a 2q32 locus may account for a proportion of such cases.

The initial G-banded cytogenetic studies done on both translocation patients indicated the existence of a common breakpoint at 2q33. However, our single-locus FISH studies strongly suggest that the breakpoints in both cases lie in distal 2q32. This discrepancy may result from the known bias in reporting of breakpoints in favor of Giemsa-pale bands (Savage 1977), or it may be that the true breakpoint is at the 2q32–2q33 band junction.

The high density of markers now available for this region of chromosome 2q allowed us to rapidly isolate a large number of genomic clones for precise delineation of the 2q breakpoints in both cleft palate cases. Early results led us to focus on the D2S311–D2S1271 interval. A CEPH YAC contig, including the distal part of this interval, has recently been described (Hadano et al. 1999). However, we chose to use the ICI library for isolation of genomic clones, because of the lower rates of chimerism in this library. The average insert size in this YAC library is 350 kb (Anand et al. 1990), and these clones are excellent FISH reagents. Formal assembly of an ICI YAC contig across the entire D2S311–D2S1271 interval was therefore not necessary, the 30 YACs isolated from this region being sufficient to allow narrowing of the translocation breakpoint interval to a small chromosomal region between D2S311 and D2S116. The close proximity of these flanking markers and the strikingly similar phenotypes of the two patients strongly suggest that it may prove that the same single gene is disrupted in both patients.

D2S311 and D2S116 are estimated to map at 207.2 Mb and 209.6 Mb, respectively, in the current version of the Location Database (LDB; Collins et al. 1996). The putative cleft-palate gene may thus already have been localized by our studies to a region of less than 2.5 Mb, although further physical studies are now needed to define this interval more precisely. We are currently isolating genomic clones containing markers mapping within this interval, for further FISH analysis. A genomic clone crossing both patients' breakpoints would be the ideal starting point for efforts to clone and characterize this cleft-palate gene. Furthermore, the availability of nonchimeric YACs proximal (31CH5, 33AC9) and distal (6HA11) to the breakpoints will allow us to walk in across the small interval by end-sequencing approaches (Riley et al. 1990). Thus a physical map across the small region of interest should be relatively straightforward to prepare. The t(2;7)(q33;p21) and t(2;11)(q33;p14) chromosomes available in the two patients described here have proved useful for confirming the marker order in this region of 2q33. The internal consistency of the separate FISH analyses with each patient and each marker is encouraging. It is hoped that the breakpoint(s), when identified, will act as a useful anchor point for the genetic map in this region.

There are several candidate genes in this region of 2q32–33. *FN1* was a particularly strong candidate, as it is localized in the developing palate in mouse embryos (Ohsaki et al. 1995; Iamaroon and Diewert 1996). Some other genes can be considered reasonable candidates because of their involvement in other morphogenetic processes, particularly *IHH* (Bitgood and McMahon 1995; Leek et al. 1997) and *IGFBP2* and *IGFBP5* (de la Rosa et al. 1994; Liu et al. 1995). Our FISH analyses, performed with YACs containing each of these genes, have effectively eliminated them from further consideration as the putative 2q32 CPO gene. Other candidates can be eliminated on the basis of their map positions relative to the D2S311–D2S116 candidate interval. Integrin alpha V (CD51) is required for proper palate formation in the mouse (Bader et al. 1998). However, its gene (*ITGAV*) maps to 2q31–q32 at position 194.2 in LDB, some 14 Mb centromeric to our candidate interval. Similarly, the homeobox genes of the *HOXD* cluster and the *DLX1–DLX2* cluster at 2q31–q32 (Simeone et al. 1994; Rossi et al. 1994) are located many megabases proximal to D2S311 and have, therefore, not been further considered in the context of the breakpoints in our two patients.

Independent support for the existence of an important cleft-palate locus at 2q32 has been obtained from our statistical analysis, showing that cytogenetic abnormalities associated with CPO are not randomly distributed and that deletions involving chromosome 2q32 are particularly likely to result in CPO. Association and/or linkage studies that make use of markers in this region and a search for chromosomal microdeletions within 2q32 in cases of nonsyndromic CPO might provide further support to our hypothesis. However, ultimate proof that 2q32 contains a CPO-causative genetic locus will require cloning of a gene whose function is disrupted by both breakpoints and the demonstration of mutations within this gene in cytogenetically normal individuals.

Acknowledgments

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Appendix

Regions Associated with Cleft Palate

All Regions Significantly Associated with Cleft Palate	Cleft-Palate Regions after Correction for Cleft Lip–Associated Cases	Cleft-Lip Regions
		1q21–25
2q32	2q31–33	
4p13–16	4p13–16	4p15–16
4q31–35	4q31–35	4q31–35
		7q34–35

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genetic Location Database, http://cedar.genetics.soton.ac.uk/public_html/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for isolated cleft palate [MIM 119540], VCFS [MIM 192430], *FN1* [MIM 135600], *IGFBP5* [MIM 146734], *IGFBP2* [MIM 146731], and *IHH* [MIM 600726])

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