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# The Gene Responsible for X-linked Cleft Palate (*CPX*) in a British Columbia Native Kindred Is Localized between *PGK1* and *DXYS1*

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## Summary

**Human craniofacial malformations are a class of common congenital anomalies in which the etiology is heterogeneous and often poorly understood. To better delineate the molecular basis of craniofacial development, we have undertaken a series of experiments directed toward the isolation of a gene involved in human secondary palate formation. DNA marker linkage studies have been performed in a large British Columbia (B.C.) Native family in which cleft palate segregates as an X-linked trait. We have examined 62 family members, including 15 affected males and 8 obligate carrier females. A previous clinical description of the clefting defect in this kindred included submucous cleft palate and bifid or absent uvula. Our recent reevaluation of the family has indicated that ankyloglossia (tongue-tie) is also a feature of X-linked cleft palate in some of the affected males and carrier females. Ankyloglossia has previously been associated with X-linked cleft palate in an Icelandic kindred in which a gene responsible for cleft palate (*CPX*) was assigned to the Xq21.3-q22 region between *DXYS12* and *DXS17*. For the B.C. kindred reported here, we have mapped the gene responsible for cleft palate and/or ankyloglossia to a more proximal position on the X chromosome. No recombination was observed between B.C. *CPX* and the DNA marker *DXS72* (peak lod score [$Z_{max}$] = 7.44 at recombination fraction [$\hat{\theta}$] = .0) localized to Xq21.1. Recombination was observed between *CPX* and *PGK1* ($Z_{max}$ = 7.35 at $\hat{\theta}$ = .03) and between *CPX* and *DXYS1* ($Z_{max}$ = 5.59 at $\hat{\theta}$ = .04). These recombination events localize B.C. *CPX* between *PGK1* and *DXYS1* in the Xq13-q21.31 region.**

## Introduction

Cleft palate is a common congenital malformation in humans. Its incidence is approximately 1/1,400 live births and varies little between populations (Leck 1984; Lowry et al. 1989). Isolated cleft palate is etiologically distinct from cleft palate associated with cleft lip (Fogh-Anderson 1942; Fraser 1980). Studies in the mouse indicate that isolated cleft palate results from failure of the secondary palatal shelves to make contact at the appropriate time for closure. The failure has been attributed to two major factors: one is reduced palatal shelf size, and the other is delayed movement of the palatal shelves from a vertical to a horizontal position (Diewert and Pratt 1981).

A complex sequence of events leads to palatal shelf elevation. Ectomesenchymal tissue arises from the cranial neural crest by about 3 wk of embryological development. Neural crest cells migrate to the primitive oral cavity and, in association with pharyngeal ectoderm, form the maxillary prominences. These extensions give rise to the two lateral palatal shelves which initially grow vertically along the sides of the intervening tongue. At approximately 8 wk, the oral cavity expands, the tongue descends, and shelf elevation occurs. Further events include shelf fusion, ossification, and soft palate–muscle formation (Ferguson 1988; Sperber 1989). It is logical to assume that mutations in genes involved in any of the processes of normal palate development could lead to clefting defects.

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Cleft palate may be a feature of malformation syndromes caused by chromosomal, Mendelian, or environmental factors, but isolated cleft palate is considered to be a multifactorial trait (reviewed in Melnick et al. 1980). There is evidence for involvement of both genetic and environmental factors, but underlying mechanisms are unknown. The complex etiology of cleft palate makes its study particularly difficult. One approach to the investigation of cleft palate is the study of the exceptional examples of cleft palate which segregate in families as single-gene disorders. Such forms of cleft palate are amenable to classical linkage analyses for gene localization, provided that adequate family material is available. There exist reports in which isolated nonsyndromic cleft palate is inherited in an autosomal dominant (Jenkins and Stady 1980; Rollnick and Kaye 1986) or X-linked manner (Lowry 1970; Rushton 1979; Rollnick and Kaye 1986; Bixler 1987; Hall 1987; Moore et al. 1987).

In one of the first reports of Mendelian-inherited cleft palate, Lowry (1970) presented a large native kindred from British Columbia (B.C.), Canada. The cleft palate in this family was described as being inherited as an X-linked trait and characterized by submucous cleft palate and bifid or absent uvula (Lowry 1970, 1971). We have undertaken a clinical reevaluation of a portion of this family and have observed that some of the affected males and carrier females present with ankyloglossia. X-linked cleft palate has been associated with ankyloglossia in at least three other families. The first family is German (Rollnick and Kaye 1986), the second is from Kentucky (Hall 1987), and the third is Icelandic (Moore et al. 1987). Linkage studies in the Icelandic kindred resulted in a provisional assignment of an X-linked locus responsible for cleft palate (*CPX*) to the Xq21.3-q22 region (Moore et al. 1987; Mandel et al. 1989). *CPX* was further localized to the interval between *DXYS12* and *DXS17* (Ivens et al. 1988). Linkage studies suggest that the cleft palate gene in the German family maps to the same region (reported in Moore et al. 1990).

Given the large size of the B.C. Native kindred, the availability of a large number of polymorphic markers on the X chromosome, and the provisional assignment of the Icelandic *CPX*, we have undertaken studies directed toward mapping and ultimately isolating the B.C. cleft palate gene. Linkage analyses were initiated with DNA polymorphic markers from the Xq21-q22 region and were later expanded to include markers from the Xq13 region. The detection of both close linkage and recombination events between several of the markers examined and B.C. *CPX* has made possible the localization of the locus responsible for cleft palate and/or ankyloglossia in the B.C. Native family.

## Material and Methods

### *Pedigree and Clinical Evaluation of Family Members*

The B.C. family consists of more than 160 living individuals in four generations. Our recent observations and previous records establish classical X-linked inheritance over five generations. In addition to the submucous cleft palate and bifid or absent uvula already reported, we have observed ankyloglossia in a significant number of affected males and carrier females. Individuals were considered to be affected if they exhibited submucous cleft palate and/or ankyloglossia. Bifid or absent uvula and/or a notch in the posterior edge of the hard palate were used as indicators of submucous cleft palate (Lowry et al. 1973; McWilliams 1991). Bifid uvula alone was not considered as adequate for assigning disease status, in light of its high frequency (10%) in the B.C. Native population (Lowry 1970).

Sixty-two family members were examined clinically (for details of the family structure, see partial pedigree in fig. 1). Nine of the 15 affected males have both submucous cleft palate and ankyloglossia. Four affected males have submucous cleft palate alone, and one affected male has ankyloglossia alone (III-5). We were unable to examine personally one of the 15 affected males (II-7), but it was observed and reported elsewhere that he has a visible incomplete cleft (Lowry 1970). It is unknown whether he has ankyloglossia. Females were considered carriers if they are clinically affected or have affected sons. Six (75%) of the eight carrier females are affected. Five of the affected females exhibit ankyloglossia, and one female (II-4) has both ankylogossia and submucous cleft palate. The disease phenotype of one individual (IV-8) is uncertain at present.

### *DNA Preparation, Southern Transfer, and Hybridization*

Blood samples (10–30 ml) were obtained from 47 family members (fig. 1). Genomic DNA was extracted using standard SDS/proteinase K digestion and phenol extraction protocols (Maniatis et al. 1989). Aliquots of 5 μg were digested with appropriate restriction enzymes for Southern blot analyses. The DNA was separated by gel electrophoresis on 0.7%–1.0% agarose gels and transferred to GeneScreenPlus (Du Pont) membrane. Prehybridization, hybridization,

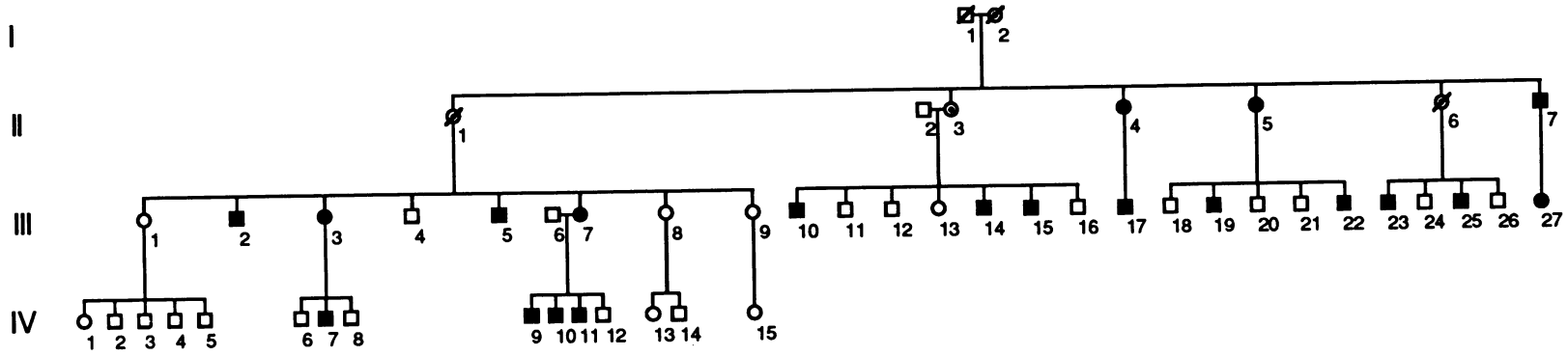


**Figure 1** Partial pedigree of B.C. *CPX* kindred. Genotype data were obtained for all living individuals shown. The disease phenotype of individual IV-8 is uncertain at present. Blackened square denotes an affected male; blackened circle denotes an affected female; unblackened square denotes an unaffected male; unblackened circle denotes an unaffected female; and circle with a dot at the center denotes an obligate carrier female. A slash (/) through the symbol indicates that the individual is deceased.

**Table 1**

**Two-Point Z's between *CPX* and X Chromosome Marker Loci**

| Locus (location) | Z at θ of .00 | .01 | .05 | .10 | .15 | .20 | .25 | .30 | .35 | .40 | .45 | $Z_{max}$ | $\hat{\theta}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *PGK1* (Xq13) | −∞ | 7.14 | 7.29 | 6.88 | 6.31 | 5.64 | 4.90 | 4.09 | 3.20 | 2.23 | 1.16 | 7.35 | .03 |
| *DXS72* (Xq21.1) | 7.44 | 7.32 | 6.84 | 6.22 | 5.58 | 4.91 | 4.21 | 3.47 | 2.69 | 1.86 | .96 | 7.44 | .00 |
| *DXYS1* (Xq21.31) | −∞ | 5.33 | 5.56 | 5.26 | 4.82 | 4.29 | 3.71 | 3.08 | 2.40 | 1.66 | .85 | 5.59 | .04 |
| *DXYS12* (Xq21.31-q21.33) | −∞ | 5.65 | 6.46 | 6.29 | 5.84 | 5.25 | 4.58 | 3.82 | 2.99 | 2.08 | 1.08 | 6.46 | .06 |
| *DXS3* (Xq21.3) | −∞ | 5.41 | 5.66 | 5.38 | 4.95 | 4.44 | 3.86 | 3.22 | 2.52 | 1.76 | .92 | 5.68 | .04 |
| *PLP* (Xq21.3-q22) | −∞ | −2.12 | .75 | 1.70 | 2.03 | 2.09 | 1.98 | 1.77 | 1.45 | 1.05 | .56 | 2.09 | .19 |
| *DXS178* (Xq21.33-q22) | −∞ | −2.25 | .64 | 1.61 | 1.96 | 2.04 | 1.96 | 1.75 | 1.44 | 1.05 | .56 | 2.04 | .20 |
| *DXS456* (Xq21-q22) | −∞ | −1.67 | −.42 | .00 | .17 | .23 | .25 | .23 | .19 | .14 | .08 | .25 | .24 |

NOTE.—Marker loci locations are from HGM10 (Keats et al. 1989; Mandel et al. 1989) and HGM10.5 (Williamson et al. 1990). Linkage data for *PGK1*, *DXS72*, *DXYS1*, *DXYS12*, *DXS3*, *PLP*, *DXS178*, and *DXS456* were contributed primarily by 30, 23, 23, 30, 24, 24, 24, and 6 meioses, respectively.

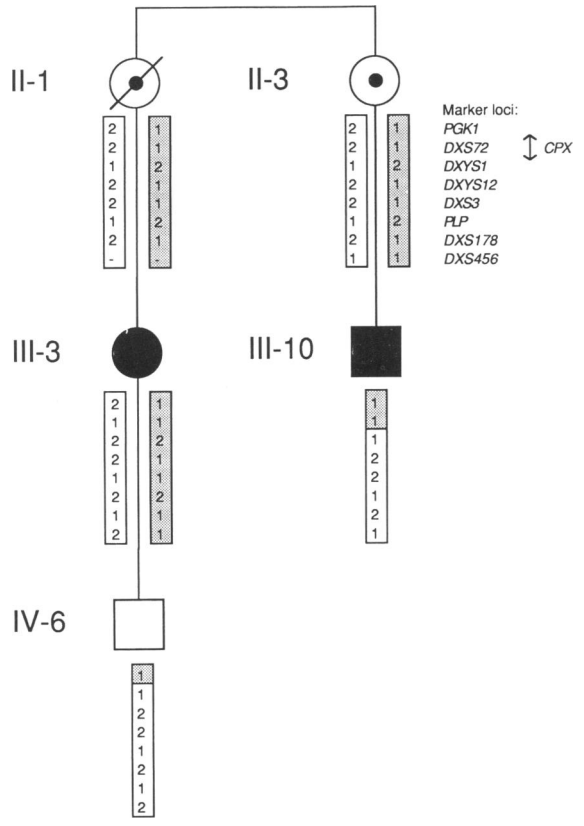


**Figure 2** Demonstration of DNA marker haplotypes for a portion of the B.C. family (II-1, II-3, III-3, III-10, and IV-6 in fig. 1). Phase was inferred so as to minimize the number of crossovers. The recombination events depicted in IV-6 and III-10 localize *CPX* distal to *PGK1* and proximal to *DXYS1*, respectively. The genotypes segregating with *CPX* are indicated by shading (▦). Other symbols are as in fig. 1.

and washes were carried out according to the manufacturer's recommendations. Autoradiography was at −70°C for 1–6 d with intensifying screens.

### *DNA Polymorphisms*

The B.C. family was tested for DNA polymorphisms at 12 loci: *DXS159* (probe cpX289, *Pst*I variants), *DXS72* (probe pX65H7, *Hin*dIII variants), *DXYS1* (probe pDP34, *Taq*I variants), *DXYS12* (probe St25-1, *Taq*I variants), *DXS3* (probe p19-2, *Msp*I variants), *DXS178* (probe p212/9, *Taq*I variants), *DXS94* (probe pXG-12, *Pst*I variants), *DXS101* (probe cX52.5, *Msp*I variants), *DXS17* (probe S21, *Taq*I variants) (Kidd et al. 1989), *PLP* (primers 4 and 5, *Acy*I variants) (P. Bridge, personal communication), *PGK1* (probe pXPGK-RI0.9, *Pst*I variants) (Williamson et al. 1990), and *DXS456* (primer XG30B, VNTR) (Luty et al. 1990; Williamson et al. 1990). DNA probes for all loci, with the exception of *PLP* and *DXS456* (described below), detected RFLPs on Southern blots. Probes were labeled by the Bethesda Research Laboratories Nick Translation System with ($^{32}$P)dCTP. Any probes containing repetitive sequences were preassociated (15 ng) with 0.5 mg of sheared human placental DNA prior to hybridization at 70°C.

The RFLP at the *PLP* locus was detected by PCR amplification with 0.4 μM each of primers 4 and 5 (P. Bridge, personal communication). Reaction conditions were altered to consist of 50–100 ng of genomic DNA, 200 μM of each dNTP, 1.1 U of Promega *Taq* polymerase, and 1 × Promega reaction buffer. Thirty cycles of 2 min at 95°C, 2 min at 55°C, and 3 min at

72°C, followed by 10 min at 72°C, were performed with a Perkin Elmer Cetus thermal cycler. The PCR product was digested with *Acy*I (Promega) and electrophoresed on a 3% NuSieve (FMC) + 1% agarose gel.

The *DXS456* locus represents a microsatellite VNTR polymorphism. Primers and reaction conditions used were as described elsewhere (Luty et al. 1990), with the following modifications: Promega reaction buffer and *Taq* polymerase (1.1 U) were used with the addition of 0.25 mM spermidine. Thirty cycles of 2 min at 95°C, 1 min at 47°C, and 2 min at 72°C, with a final 72°C incubation for 10 min, were performed with a Perkin Elmer Cetus thermal cycler.

### *Linkage Analyses*

Linkage analyses were performed using the MLINK, ILINK, and LINKMAP programs of the LINKAGE package (v5.10) (Lathrop et al. 1984, 1985). Two-point linkage analyses between *CPX* and marker loci were conducted using MLINK. Lod score ($Z$) values were calculated at various recombination fraction ($\theta$) values (table 1). The maximum two-point $Z$'s ($Z_{max}$) and corresponding $\hat{\theta}$'s (i.e., as in table 1) were determined using ILINK (table 1). The frequency of the *CPX* mutant allele was set at .0005. This value is intermediate between that which Moore et al. (1987) used for *CPX* in the Icelandic kindred linkage analysis and that which Weatherley-White et al. (1972) reported for submucous cleft palate. The penetrance of *CPX* was set at 1.0 in males and .75 in heterozygous females. Individuals were scored as affected or unaffected according to clinical diagnosis. All deceased individuals were scored as unknown.

Multipoint linkage analysis was performed using LINKMAP. Location scores (twice the natural logarithm-of-the-odds ratio) were calculated for all possible positions of B.C. *CPX* relative to a map of the four most closely linked marker loci deduced from the two-point analyses. The order of the marker loci and the distances between them were assumed to be fixed; the locus order used was *PGK1–DXS72–DXYS1–DXYS12*, with respective $\theta$'s of .040, .044, and .048. The $\theta$'s are approximations based on combined data from Keats et al. (1989, 1990) and Puck et al. (1991). Genetic distances (in cM) were calculated using Haldane's (1919) mapping function.

## Results

Genotypes for 12 polymorphic markers from the Xq13-q22 region were determined for the purpose of linkage analyses. Four markers—*DXS159, DXS94, DXS101,* and *DXS17*—were uninformative. The eight remaining markers were fully or partially informative. Genotype data and deduced haplotypes for a portion of the family, which includes two recombinant individuals (IV-6 and III-10), are presented in figure 2. The order of the marker loci, proximal to distal, is that shown in figure 2 and table 1; the positions and relative orders for *PLP, DXS178,* and *DXS456* are provisional. The haplotype apparently segregating with *CPX* is consistent throughout the partial pedigree (fig. 1).

$Z$'s for two-point disease-to-marker analysis are summarized in table 1. The number of informative meioses for each marker is indicated. No recombination was observed between *CPX* and *DXS72* ($Z_{max}$ = 7.44 at $\hat{\theta}$ = .0) localized to Xq21.1. A single recombination event between *CPX* and the marker *PGK1* ($Z_{max}$ = 7.35 at $\hat{\theta}$ = .03) places *CPX* distal to *PGK1* (fig. 2). This critical crossover was detected in an unaffected male (IV-6). Seven recombination events were observed between *CPX* and markers distal to *DXS72*. One crossover was detected between *CPX* and *DXYS1* ($Z_{max}$ = 5.59 at $\hat{\theta}$ = .04) and places *CPX* proximal to *DXYS1* (fig. 2). A second recombination event was observed between *CPX* and *DXYS12* ($Z_{max}$ = 6.46 at $\hat{\theta}$ = .06). Both of these crossovers were detected in affected males (III-10 and IV-10, respectively). Four of the recombination events occurred between *CPX* and both *PLP* and *DXS178,* and the final event occurred between *CPX* and *DXS456*. No double crossover events were observed in the interval examined.

Multipoint linkage analysis supports the *CPX* localization suggested by the recombination events described above. The location score results and fixed-loci map are presented in figure 3. The multipoint $Z$ (= location score/2ln[10]) is maximum at the position of *DXS72* (multipoint $Z$ = 9.56), in the interval between *PGK1* and *DXYS1*. The odds against *CPX* localization are greater than 100:1 (i.e., the value generally considered meaningful for exclusion of an interval) in all other intervals tested, except that proximal to *PGK1*. At −0.03 cM, the odds against *CPX* localization are just 24:1, indicating another possible, though less likely, position for *CPX*. The support interval (1 lod difference from the maximum-likelihood estimate of location) for *CPX* at *DXS72* is equivalent to the region between *PGK1* and *DXYS1* (fig. 3).

## Discussion

DNA marker linkage studies have proved successful in regionally localizing a large number of X-linked

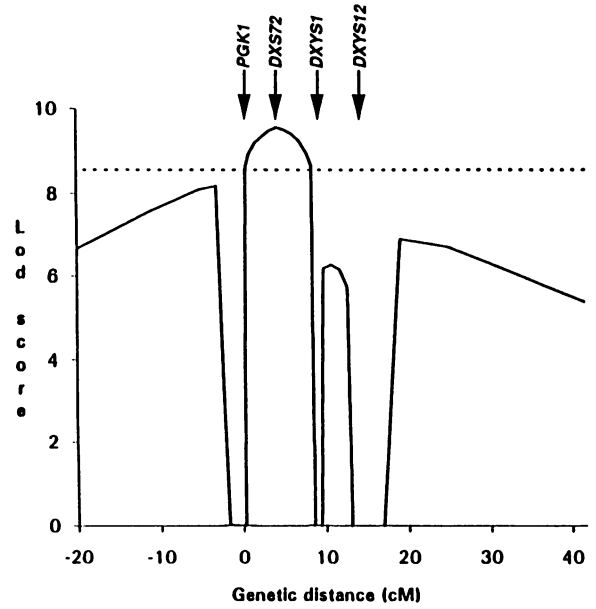


**Figure 3** Multipoint linkage results for *CPX* vs. a fixed loci map. The position of *PGK1* was arbitrarily set at 0 cM. The overall $Z_{max}$ occurs at the *DXS72* position, between *PGK1* and *DXYS1*. The dashed line is drawn 1 lod interval below the maximum-likelihood estimate of *CPX* location.

disease genes (Mandel et al. 1989). We have localized, by linkage analyses, the locus responsible for cleft palate and/or ankyloglossia in a B.C. Native kindred to the Xq13-q21.31 region between *PGK1* and *DXYS1*. The B.C. *CPX* localization is consistent with one of two possible locations for Icelandic *CPX*, on the basis of multipoint linkage analysis. Multipoint linkage analysis in the Icelandic kindred positioned *CPX* either 5 cM proximal or 7 cM distal to the *DXYS1* locus. Analyses of critical recombinants in the Icelandic family supported the distal location and placed *CPX* between *DXYS12* (proximal marker) and *DXS17* (distal marker) (Ivens et al. 1988).

In the B.C. kindred, two recombination events observed in affected males place B.C. *CPX* proximal to *DXYS12*. One crossover event was between *CPX* and all informative markers distal to, and including, *DXYS1*. The second crossover event occurred between *CPX* and all informative markers distal to, and including, *DXYS12*. In the second individual, *DXYS1* was uninformative. The apparent inconsistency in the localization of the CPX gene(s) in the Icelandic and B.C. kindreds may be attributed to genetic heterogeneity. An alternate explanation is that disease status or marker genotypes were assigned incorrectly in one or more individuals.

The cleft palate and/or ankyloglossia phenotype appeared to be fully penetrant in the male members of the B.C. family. In no instance was the disease-associated haplotype observed in an unaffected male. In carrier females, cleft palate and/or ankyloglossia was 75% penetrant.

The expressivity of cleft palate and ankyloglossia is highly variable in both males and females. This observation is consistent with the phenotypic variability described in the three previously reported families with X-linked cleft palate and ankyloglossia (Rollnick and Kaye 1986; Hall 1987; Bjornsson et al. 1989). Differences in severity of *CPX* expression could be due to environmental factors, other genetic factors, and/or, in carrier females, random X inactivation.

As suggested by Hall (1987), ankyloglossia may be a useful diagnostic marker for X-linked cleft palate. In the B.C. kindred, ankyloglossia was present in 67% of affected males and in 75% of carrier females. Ankyloglossia is not usually associated with isolated (i.e., multifactorial) cleft palate. It is unlikely, though, that careful examination for microforms of cleft palate has been conducted in most ankyloglossia cases. Tongue movement is clearly involved in palate development, but the significance of the phenotypic association between the X-linked form of cleft palate and ankyloglossia is not clear.

Bifid or absent uvula was not used as a sufficient indicator of disease status in the B.C. kindred, because of the high frequency (10%) of such anomalies in the B.C. Native population (Lowry 1970). However, bifid or absent uvula in the B.C. kindred was observed in 60% of affected males and in 38% of carrier females, compared with 9% of unaffected males and 0% of noncarrier females. Bifid or absent uvula has also been observed in two other families with X-linked cleft palate and ankyloglossia (Rollnick and Kaye 1986; Hall 1987).

There are several reports of viable males with Xq21 deletions encompassing a large part of the *CPX* candidate region. Clinical symptoms associated with these deletions may include choroideremia, mental retardation, and deafness. Female carriers of the deletions are asymptomatic, with the exception of retinal changes characteristic of the choroideremia carrier state (Tabor et al. 1983; Hodgson et al. 1987; Nussbaum et al. 1987; Cremers et al. 1989; Merry et al. 1989). The only documented Xq21 deletion individual (N.P.) (Tabor et al. 1983) with cleft palate has unilateral cleft lip and palate. Cleft palate in association with cleft lip is believed to be etiologically distinct from isolated cleft palate (Fogh-Anderson 1942; Fraser 1980).

There are several possible explanations for the ob-

servation of Xq21-region deletions in the absence of cleft palate. First, it would seem that the *CPX* mutation is not an amorph (loss-of-function mutation). This suggestion is consistent with the apparent dominant nature of the mutation. The cleft palate and/or ankyloglossia phenotype may be the result of an alteration in the *CPX* product. Second, it is possible that *CPX* is located outside the region encompassed by the Xq21 deletions. The distal breakpoint of the largest male viable deletion (individual RvD) is just centromeric to *DXS17,* and the proximal breakpoint is between *DXS72* and *PGK1* (Cremers et al. 1989). It would follow, then, that *CPX* is located proximal to *DXS72*. Third, the mutation in *CPX* may predispose for cleft palate and/or ankyloglossia but may not necessarily be causative.

In an effort to localize B.C. *CPX* more precisely, we are currently examining linkage with additional polymorphic markers from the region between *PGK1* and *DXYS1*. Fine mapping of *CPX* will facilitate experiments directed toward gene isolation. Elucidation of the molecular defect underlying cleft palate and/or ankyloglossia in the B.C. family may provide insight into the nature of genes and gene products involved in the more frequently occurring, non-Mendelian cleft palate in the general population. Furthermore, it may lead to the study of a class of genes involved in craniofacial development and/or genes that confer susceptibility to environmental factors affecting craniofacial development.

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