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Partial Triplication of mtDNA in Maternally Transmitted Diabetes Mellitus and Deafness

To the Editor:

Maternally inherited diabetes and deafness (MIDD) is a recently recognized subtype of diabetes mellitus (DM) that is associated with mtDNA mutations (Maassen et al. 1997). The first mtDNA defect described for MIDD was a deletion associated with a duplication of the mtDNA in a family presenting DM and deafness over three generations (Ballinger et al. 1992, 1994). Subsequent to this observation, a mutation in nucleotide (nt) 3243 was reported in several pedigrees presenting DM and deafness (Reardon et al. 1992; van den Ouweland et al. 1992; Kadowaki et al. 1993). We report a partial tandem triplication of 9.2 kb in one member of a family presenting MIDD associated with a tandem duplication of 4.6 kb.

In 1966, a 44-year-old man (II-7) of Italian origin was hospitalized for insulin-dependent DM and hearing loss. In 1973, his nephew (III-2), who was born in 1932, was hospitalized for non–insulin-dependent DM and deafness. At that time, the morbid association led to a study of the pedigree (fig. 1), which showed transmission of DM and deafness over four generations, with a total of 13 affected individuals (Kressmann 1976). Seven individuals from the pedigree (III-3, III-4, IV-1, IV-2, IV-3, IV-4, and IV-5) were examined by clinicians. The clinical history was the same for all affected patients: the first manifestation was deafness, beginning at 20–30 years of age, with a rapid and severe increase in bilateral sensory hearing loss. DM developed later in the 3d decade, and insulin was required either immediately or at a later date. At that time, the individuals from the fourth generation, who were <20 years of age, presented no deafness or DM. No pedigree member had ptosis, ophthalmoplegia, or muscle weakness. Recently, the maternal inheritance pattern of DM and deafness in this family

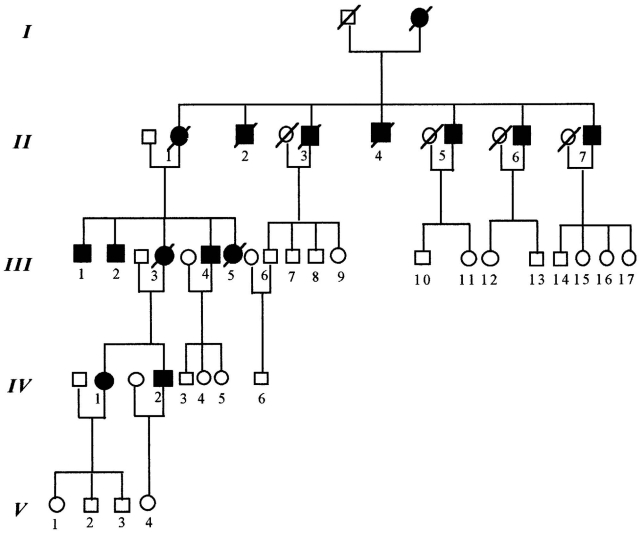

Figure 1 Pedigree of family analyzed in this study. Unblackened symbols indicate unaffected individuals, and blackened symbols indicate affected individuals. Nine family members (II-7, III-2, III-3, III-4, IV-1, IV-2, IV-3, IV-4, and IV-5) were examined in 1973.

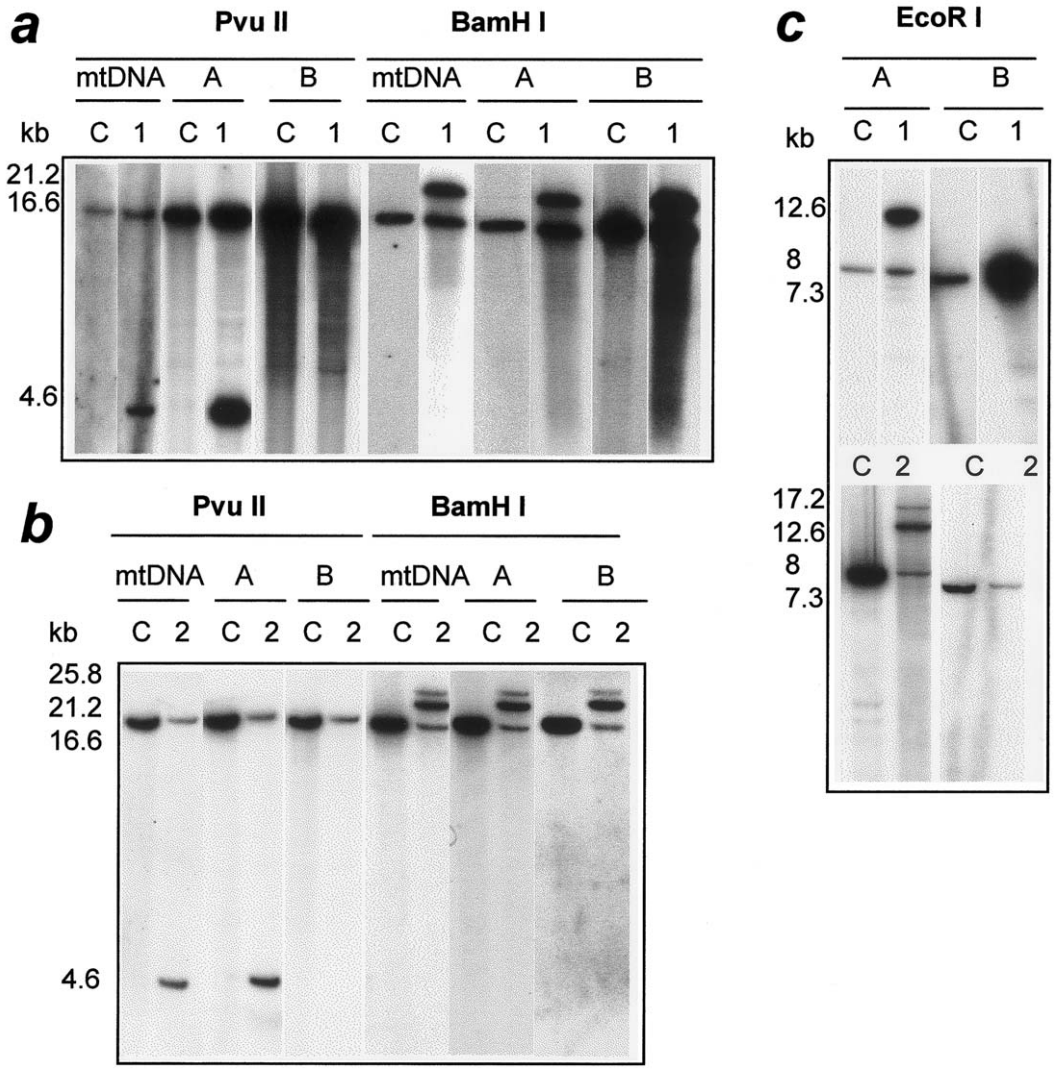

Figure 2 *a,* mtDNA of patient 1, digested with *Pvu*II or *Bam*HI and probed with an mtDNA probe, probe A and probe B. The mtDNA showed additional fragments of 4.6 kb (*Pvu*II digest) and 21.2 kb (*Bam*HI digest), respectively, that are consistent with a partial duplication of 4.6 kb. *b,* mtDNA of patient 2. A supplementary band of 25.8 kb was visualized with *Bam*HI digestion. This band was detected after hybridization with probe A included in the duplicated region (nts 2630–3353) and with probe B not retained in the duplicated segment (nts 7392–8351), thus ruling out circular deleted monomers or dimers. *c,* Digestion with *Eco*RI. For the control DNA, the expected fragments of 8, 7.3, and 1 kb are shown (the 1-kb band is not visualized). The mtDNA of patient 1 shows a supplementary band of 12.6 kb labeled with probe A but not with probe B, corresponding to the partially duplicated molecule. For patient 2, one additional band of 17.2 kb was evidenced with probe A but not with probe B. This is interpreted as a new mtDNA species harboring a tandem repetition of the 4.6-kb duplicated sequence of patient 1.

was noticed, and three patients were examined again by clinicians. Patient 1 (IV-1), 40 years old, and patient 2 (III-1), 65 years old, presented severe deafness and DM that, with time, required insulin. Patient 3 (IV-2), 36 years old, had moderate bilateral sensory hearing loss and subnormal glucose tolerance.

Histopathological studies of the skeletal muscle biopsy specimens from patients 1 and 2 showed no ragged red fibers, a complex IV enzymatic deficiency in a few fibers, and very limited lipid storage on electron microscopy. Neither mitochondrial hyperplasia nor inclusions were observed. No abnormalities were observed for patient 3.

Total DNA was extracted from the muscle biopsy specimens and blood of the three patients. The search for the mtDNA mutation in the $tRNA^{leu(UUR)}$ gene at nt 3243 was performed in accordance with a protocol described elsewhere (Ciafaloni et al. 1991). For Southern blotting, 5 μg of total muscle DNA and 10 μg of blood DNA were digested with restriction enzyme *Pvu*II (nt

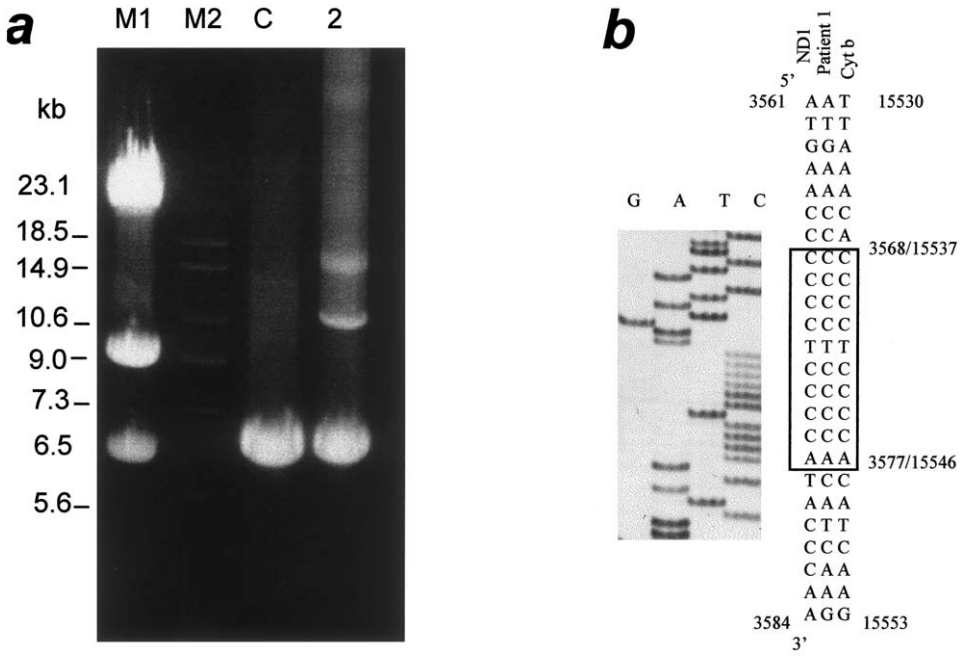

Figure 3 *a,* PCR products obtained after amplification with primers 5 and 6. A 6.8-kb band was detected in control DNA. For patient 2, two supplementary bands of 11.4 kb and 16 kb were present, thus confirming the results of the Southern blot analysis, with regard to the existence of mtDNA molecules linked to one (duplicated species) or two (triplicated species) rearranged molecules of 4.6 kb. "M1" and "M2" indicate the molecular-weight markers. Lane M1, Phage λ, digested with *Hind*III. Lane M2, Raoul. Lane C, Control DNA. Lane 2, Patient 2. *b,* Sequence across the duplication junction of patient 1. The sequencing of the cloned 369-bp PCR products obtained after amplification with primers 3 and 4 showed a normal sequence for region 3274–3577. Subsequent nts corresponded exactly to region 15547–15600. The duplication junction is a perfect direct repeat of 10 nts, located in regions 3568–3577 (ND1 gene) and 15537–15546 (Cyt b gene). The boxed region indicates the 10-bp perfect direct repeat. The normal sequences of ND1 and Cyt b correspond to the left and right sequences, respectively. The same 369-bp PCR products were sequenced in patients 2 and 3, and identical results were obtained.

2650), *Bam*HI (nt 14258), or *Eco*RI (nts 4121, 5274, and 12640), in accordance with the manufacturer's recommendations; were separated by gel electrophoresis; and were blotted onto nylon membrane (Hybond N^+, Amersham). Hybridization was performed with a random-primed ^{32}P-labeled mtDNA probe (Lutfalla et al. 1985) and with two random-primed ^{32}P-labeled mtDNA probes derived from PCR products spanning nts 2630–3353 (probe A) and nts 7392–8351 (probe B). Quantification was performed with a Phosphor Imager (Molecular Dynamics) by scanning of the nylon filters of the *Bam*HI digests hybridized with probe B. PCR analyses of the duplicated region were performed on muscle and blood samples by use of two different couples of primers (primer 1, nts 2630–2650, 5′-GAA TGG CTC CAC GAG GGT TC-3′, and primer 2, nts 16255–16274, 5′-CCT AGT GGG TGA GGG GTG GC-3′; primer 3, nts 3274–3293, 5′-ACA GTC AGA GGT TCA ATT CC-3′, and primer 4, nts 15581–15600, 5′-GGG ACG GAT CGG AGA ATT GT-3′). Amplification conditions were 30 cycles of 1 min at 93°C, 1 min at 62°C (primers 1 and 2) or at 55°C (primers 3 and 4), and 2 min at 72°C, with 2.5 U of *Taq* polymerase (Promega). The PCR products obtained with primers 1 and 2 were analyzed with restriction enzymes *Bcl*I (nts 3658, 7657, 8591, and 11921), *Eco*RI (nts 4121, 5274, and 12640), *Eco*RV (nts 3179, 6734, and 12871), *Kpn*I (nts 2573, 16048, and 16121), and *Xho*I (nt 14955). The 369-bp PCR fragment obtained after amplification with primers 3 and 4 was cloned into the pGEM-T vector (Promega) and was used as a template for dideoxy sequencing using the T7 sequencing kit (Pharmacia), in accordance with the manufacturer's specifications, to reveal the duplication junction. To amplify all length variants of the mtDNA molecules (normal, duplicated, and triplicated) in patient 2, a long PCR was performed with a DNA thermal cycler (Robocycler, Stratagene) and the Expand Long PCR Template PCR system (Boehringer Mannheim), by use of the manufacturer's recommendations modified as described elsewhere (Fromenty et al. 1996). The amplification conditions were 35 cycles for 30 s at 93°C, 30 s at 66°C, and 17 min at 68°C. The primer pair comprised primer 5 (forward primer), nts 13949–13972, 5′-CCT ATC TAG GCC TTC TTA CGA

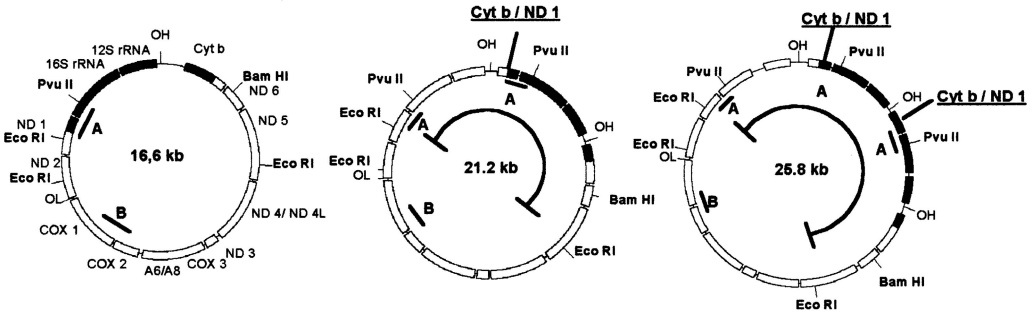

Figure 4 Schematic representation showing the normal mitochondrial genome (16.6 kb), the partially duplicated mtDNA molecule (21.2 kb) found in the three patients, and the abnormal molecule harboring the triplication (25.8 kb) in patient 2. The *Pvu*II, *Bam*HI, and *Eco*RI sites and the locations of probe A (nts 2630–3353) and probe B (nts 7392–8351) are indicated. The curved lines indicate the regions corresponding to the *Eco*RI digests of 12.6 kb and 17.2 kb, for the Southern blot analysis of patient 2. Black boxes indicate the genes involved in the rearrangement in the normal molecule and the duplicated or triplicated fragments in the rearranged genomes.

GCC-3′, and primer 6 (reverse primer), nts 4207–4186, 5′-GTA ATG CTA GGG TGA GTG GTA G-3′.

None of the patients carried the pathogenic point mutation at nt 3243. On the other hand, the results of Southern blot analysis of muscle DNA from patients 1 and 3, digested with restriction enzymes *Pvu*II and *Bam*HI, were consistent with a partial duplication of a 4.6-kb region of mtDNA that included the *Pvu*II restriction site (nt 2650) but not the *Bam*HI site (nt 14258) (fig. 2*a*). Southern blot analysis of skeletal muscle DNA from patient 2 unexpectedly revealed an additional 25.8-kb band on *Bam*HI digestion (fig. 2*b*), which could correspond to either (1) an undigested circular deletion monomer or dimer, (2) a second, larger duplicated molecule, or (3) an additional insert of 4.6 kb corresponding to a partially triplicated molecule. Hybridization of the 25.8-kb band with a probe not included in the duplication (probe B) ruled out a circular deletion monomer or dimer. The possibility of a second species duplication also was ruled out, because an abnormal band >4.6 kb was not detected with the *Pvu*II digest, and only one band was obtained by PCR when primers 3 and 4 were used. The possibility of an mtDNA triplication in patient 2 was confirmed by digestion of the DNA, with *Eco*RI, which gave two additional fragments, compared with that of the control (fig. 2*c*): one fragment, of 12.6 kb, corresponded to the partial duplication also found in patient 1, and the other, of 17.2 kb, was consistent with an mtDNA molecule linked to two partially duplicated molecules. The triplication was confirmed further by means of long PCR using primers 5 and 6 (fig. 3*a*). PCR analysis and sequencing showed that the breakpoint junction was located between the ND1 gene and the cytochrome (Cyt) b gene at a 10-bp perfect direct repeat (fig. 3*b*). These results indicate the presence of three species of mtDNA molecules in patient 2: a normal molecule (16.6 kb), a rearranged molecule (21.2 kb) that contains an additional 4.6-kb fragment corresponding to a partial tandem duplication, and a rearranged molecule (25.8 kb) that contains two copies of the 4.6-kb fragment corresponding to a partial triplication (fig. 4). The proportion of duplicated mtDNA in muscle was 42% for patient 1 and 61% for patient 2. The proportion of triplicated molecules was only 6% for patient 2. In blood, the proportion of duplicated molecules was 52% for patient 1 and 67% for patient 2. No triplicated molecules were detected in the blood.

Partial triplication of human mtDNA is an extremely

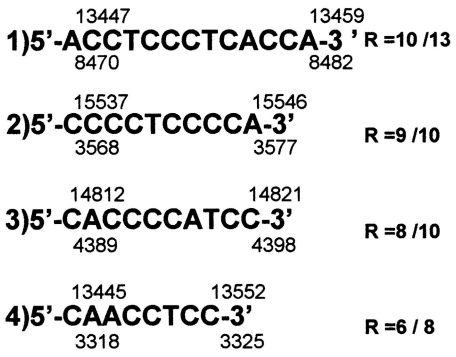

Figure 5 DNA sequences of perfect direct repeats located across breakpoint junctions of the mtDNA reported in the literature and showing a polypyrimidine tract (*1*) in the common deletion (Schon et al. 1989); (*2*) in the family described here and in two other cases of duplication/deletion associated with myopathy (Fromenty et al. 1996; Manfredi et al. 1997); (*3*) in a case of duplication/deletion associated with DM (Ballinger et al. 1992, 1994); and (*4*) in a duplication associated with DM and myopathy. "R," reported as 6/8 in (*4*), corresponds to a ratio of 13/18 if the entire imperfect direct repeat of 18 nts is considered (Dunbar et al. 1993). "R" indicates the number of pyrimidines in the direct repeat of the light-strand DNA template.

rare event. Only two cases have been reported previously: one in cell culture (Holt et al. 1997) and a second identified from autopsy material from a clinically asymptomatic individual (Tengan and Moraes 1998). The molecular mechanisms leading to large-scale rearrangements have not been well characterized yet, and models of slippage mispairing or illegitimate recombination events have been proposed (Shoffner et al. 1989; Poulton et al. 1993). Nevertheless, the origin of slippage mispairing still remains elusive. Like other reported examples of large-scale rearrangements (fig. 5), our direct repeat harbors a long polypyrimidine (L strand)/polypurine (H strand) sequence. We suggest that the second direct repeat (polypyrimidine/polypurine tract) could interact with the first direct repeat to form a triple helix (H DNA) and leads thereafter to the first tandem duplication. The repetition of this mechanism then could lead to the triplication.

A major cause of diabetes in DM and deafness seems to be a decrease in ATP production in pancreatic β cells that could be responsible for the decrease in insulin secretion (Dukes et al. 1994; Gerbitz et al. 1996). Under normal physiological conditions, the increase in blood glucose concentration results in an increase in ATP production in pancreatic β cells, which in turn leads to the closure of K^+ channels located in the cell membrane. This closure induces a membrane depolarization and the opening of voltage-dependent Ca^{2+} channels. The influx of Ca^{2+} into β cells then stimulates insulin exocytosis. In DM and deafness, gene defects lead to an oxydative phosphorylation disturbance and eventually to decreased ATP production. The pathogenic role of duplicated or triplicated mtDNA molecules in this context is difficult to assess, because all of the mtDNA information content is present in these rearranged molecules. In addition, some experiments have indicated a pathogenic role only for mtDNA deletions (Manfredi et al. 1997). Nevertheless, like others (Dunbar et al. 1993), we have not detected any deleted molecules, and we cannot exclude a possible respiratory-chain impairment secondary to duplicated or triplicated molecules. Indeed, an increase in lactate production in cell cultures that harbor duplicated and triplicated mtDNA has been demonstrated (Holt et al. 1997).

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Reply to Inglehearn

To the Editor:

In our article "Localization of a Novel X-Linked Progressive Cone Dystrophy Gene to Xq27: Evidence for Genetic Heterogeneity" (Bergen and Pinckers 1997), we presented evidence favoring a location, on Xq27, for a cone dystrophy gene. This localization is questioned by Dr. Inglehearn (1998) in his letter "LOD Scores, Location Scores, and X-Linked Cone Dystrophy." Although Dr. Inglehearn makes a good (methodological) point, we feel that the majority of his criticism is not justified.

Clearly, as Dr. Inglehearn states correctly, figure 2 in our previous article (Bergen and Pinckers 1997) shows a picture of the multipoint location scores rather than of the multipoint LOD scores. Although the presentation of location scores instead of multipoint LOD scores is not wrong in itself, it is rather unconventional and therefore confusing. Thus, we agree that, with regard to a multipoint location score of 10.8, the calculated multipoint LOD score is indeed 2.35. Obviously, for X-chromosomal disorders, the latter score is still considered to be significant.

Subsequently, Dr. Inglehearn calculates, on the basis of the data presented, multipoint (maximum?) LOD scores of 3.38 and 2.46 at DXS998, using different LOD-score strategies. Unfortunately, additional calculations for other markers are not given. Both these LOD scores for DXS998 are higher than the true multipoint LOD scores calculated by us (maximum LOD score [Z_{max}] of 2.35). Thus, in our article (Bergen and Pinckers 1997), our calculation of LOD scores and our choice of parameters were in fact very conservative. Therefore, the assertion by Dr. Inglehearn (1998) that "these data do indeed suggest a locus for X-linked cone dystrophy in this region but with rather less significance than Bergen and Pinckers have stated" (p. 900) is not justified. Most likely, the true findings for the Z_{max} score at DXS998 are somewhere within the range 2.35–3.38.

Dr. Inglehearn states that a second weakness of the article is the order and placement of markers used in the multipoint linkage analysis. However, this assertion is based on out-of-date and incomplete genetic maps of the region, as indicated by the references to literature published in 1992 and 1994 (NIH/CEPH Collaborative Mapping Group 1992; Gyapay et al. 1994), and therefore is not justified. Much more recent and up-to-date consensus maps (Dib et al. 1996) place DXS998 ~15 cM from the distal tip of the X chromosome and at least 7 cM proximal to the red cone pigment (RCP)/green cone pigment (GCP) gene cluster.

In addition, in our article (Bergen and Pinckers 1997), data on two additional markers, DXS297 and DXS1123, are presented. Both DXS297 and DXS1123 reveal higher (maximum two-point) LOD scores of 2.54 and 2.60, respectively, without recombination with COD2, but these markers are ignored in the comments by Dr. Inglehearn. Most likely, on the basis of recombination counting, haplotype analysis, and marker-to-marker analysis, both DXS297 and DXS1123 are part of a cosegregating haplotype, together with DXS998 and COD2. Although DXS297 and DXS1123 are not present on the CEPH/Généthon consensus maps, at least two independent reports in the literature (Richards et al. 1991; Donnelly et al. 1994) place DXS297 proximal to the fragile X site, which is located on Xq27.3 (Dib et al. 1996). Similar, although somewhat weaker, evidence can be found for DXS1123. In contrast, the RCP/GCP gene cluster is located on Xq28. In conclusion, there is