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Myotonic Dystrophy: Size- and Sex-dependent Dynamics of CTG Meiotic Instability, and Somatic Mosaicism

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Summary

Myotonic dystrophy (DM) is a progressive neuromuscular disorder which results from elongations of an unstable (CTG)n repeat, located in the 3′ untranslated region of the DM gene. A correlation has been demonstrated between the increase in the repeat number of this sequence and the severity of the disease. However, the clinical status of patients cannot be unambigously ascertained solely on the basis of the number of CTG repeats. Moreover, the exclusive maternal inheritance of the congenital form remains unexplained. Our observation of differently sized repeats in various DM tissues from the same individual may explain why the size of the mutation observed in lymphocytes does not necessarily correlate with the severity and nature of symptoms. Through a molecular and genetic study of 142 families including 418 DM patients, we have investigated the dynamics of the CTG repeat meiotic instability. A positive correlation between the size of the repeat and the intergenerational enlargement was observed similarly through male and female meioses for ≤0.5-kb CTG sequences. Beyond 0.5 kb, the intergenerational variation was more important through female meioses, whereas a tendency to compression was observed almost exclusively in male meioses, for ≥1.5-kb fragments. This implies a size- and sex-dependent meiotic instability. Moreover, segregation analysis supports the hypothesis of a maternal as well as a familial predisposition for the occurrence of the congenital form. Finally, this analysis reveals a significant excess of transmitting grandfathers partially accounted for by increased fertility in affected males.

Introduction

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disorder characterized by a highly variable clinical phenotype including myotonia, progressive muscle weakness, and cataract. It is the most common form of adult muscular dystrophy (Harper 1989). DM can also occur as a severe neonatal form only when transmitted by a carrier woman. However, <10% of affected mothers give birth to congenitally affected infants (Koch et al. 1991). The major clinical features of the congenital form include neonatal hypotonia, facial diplegia, respiratory distress, and weakness of sucking and swallowing, but no cataract and myotonia which are characteristic of the adult form. Hydramnios and reduced fetal movements during pregnancy, together with talipes, are frequent. Mortality is high, and most survivors have motor and mental retardation and later develop the classic form of the disease (Vanier 1960; Harper 1989).

All DM mutations characterized to date appear as unstable elongations of a small fragment which contains a tandem repetition of a CTG motif in the 3′ untranslated region of a protein kinase–like gene on chromosome 19q13.2-13.3 (Aslanidis et al. 1992; Brook et al. 1992; Buxton et al. 1992; Fu et al. 1992; Harley et al. 1992*a;* Mahadevan et al. 1992). It has been shown that the fragment is polymorphic in the normal population (Brook et al. 1992; Caskey et al. 1992; Harley et al. 1992*b;* Mahadevan et al. 1992). In addition, the enlarge-

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ment of this mutation from one generation to another seems to be correlated with age at onset and increasing severity of the disease in successive generations, thus giving legitimacy to the phenomenon known as "anticipation" (Aslanidis et al. 1992; Brook et al. 1992; Buxton et al. 1992; Caskey et al. 1992; Fu et al. 1992; Harley et al. 1992*a*, 1992*b*; Harper et al. 1992; Mahadevan et al. 1992; Tsilfidis et al. 1992; Yamagata et al. 1992). However, because the length of the CTG repeat in lymphocytes and the phenotype of the DM patients are not strictly correlated, it remains difficult to predict the prognostic and diagnostic value of the size of the repeat (Fu et al. 1992; Harley et al. 1992*b*; Mahadevan et al. 1992; Tsilfidis et al. 1992). Moreover, the exclusive maternal inheritance of the congenital form remains unexplained.

This raises several questions: (1) Could somatic mosaicism explain the absence of a strict correlation between the size of the mutation observed in patient lymphocytes and the severity of the disease? (2) Are some women already "committed" to transmit a DM chromosome which will necessarily induce a congenital form? (3) Is the meiotic instability of the DM mutation dependent on the sex of the affected parent and/or on the size of the parental CTG repeat?

In this context, we performed a segregation analysis on 142 families. In addition, we characterized the DM mutation in different tissues from one individual and in lymphocytes from 329 patients belonging to 117 DM families, mostly French. This study was carried out using probes cDNA25 (Buxton et al. 1992) and p5B1.4 (Shelbourne et al. 1992) and using PCR analysis with oligos 101 and 102 (Brook et al. 1992).

Subjects and Methods

Families

Blood samples of 570 individuals from 116 DM families including 326 affected patients were collected. They were all referred to us for molecular analysis in a context of presymptomatic and/or prenatal diagnosis. According to severity and age at onset, we have grouped the patients into three different classes: (1) class M—individuals presenting in later life with minimal features of the disease (cataracts and/or baldness) and asymptomatic obligate carriers (grandparents), (2) class A—classic adult-onset type, and (3) class C—congenitally affected newborns exhibiting signs of the disease (respiratory distress, hypotonia, facial diplegia, weakness of sucking and swallowing, etc.) during the neonatal period or children with early severe childhood onset. Additional clinical and pedigree information was also obtained for remote relatives and for 26 other DM families. Molecular and pedigree analyses were conducted on the basis of nonquestionable status, and results are given according to the clinical status reliability.

Southern Blot Experiments

DNA was prepared from whole blood and from lymphoblastoid cell lines according to a method reported elsewhere (Henry et al. 1984). Restriction-enzyme digests were performed according to the manufacturers' instructions, and fragments were resolved by 1% agarose gel electrophoresis.

Probes were labeled with [α-32P] dCTP by random priming (Boehringer Mannheim). The DM mutation was detected by using probes cDNA25 (Buxton et al. 1992) and p5B1.4 (Shelbourne et al. 1992) with the restriction enzymes *Eco*RI and *Bam*HI, respectively. CTG expansions were measured to the midpoint of smears. In order to limit intrafamilial variation of the estimated size of the mutation, DNA samples from sibs and parents were comigrated in the same gel and were transferred onto the same membrane.

PCR Analysis

PCR analysis was performed to study both the non-DM individuals and the DM patients showing a small increase of the CTG region (<80 repeats) or when no increase was detectable on Southern blots. PCR was carried out in a total volume of 50 ml with 50 ng of DNA; primers at 0.5 mM in 50 mM KCl; 10 mM Tris pH 8.3; 1.5 mM $MgCl_2$; 0.01% gelatin; dATP, dCTP, dGTP, and dTTP at 300 mM each (Pharmacia); and 0.5 units of *Taq* polymerase (Perkin Elmer Cetus), for 30 cycles at 94°C denaturation (1 min), 62°C annealing (1 min), and 72°C extension (1 min) in a Perkin Elmer Cetus 96-well thermocycler. Initial denaturation was 5 min at 94°C, and the last extension was 5 min at 72°C. Primers used were oligos 101 and 102, described elsewhere (Brook et al. 1992). The PCR fragments produced were separated through 20-cm vertical 6% polyacrylamide gels (H5 apparatus; BRL), after overnight migration (12 mA) at 4°C. Allelic fragments were analyzed after ethidium bromide staining.

Results and Discussion

Polymorphism of the CTG Repeats in Normal French Individuals

In order to assess length variation within the CTG repeat, we analyzed the PCR fragments produced with

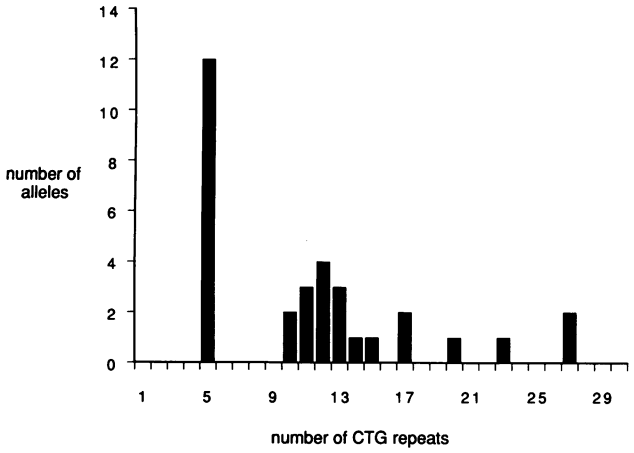

Figure 1 Distribution of alleles within the normal population. The *X*-axis represents the number of CTG repeats per chromosome, and the *Y*-axis represents the number of alleles observed with this repeat length. The total number of chromosomes analyzed was 32.

oligos 101 and 102 in 16 unrelated normal French individuals. Eleven distinctively sized alleles, ranging from 128 bp to 194 bp, were identified, suggesting a variation of the number of repeats, from 5 to 27. The distribution of the different alleles among normal individuals is shown in figure 1.

These data on normal French individuals are similar to the data previously reported by Brook et al. (1992), Mahadevan et al. (1992), and Davies et al. (1992), on individuals of other ethnic Caucasian origins. The majority of individuals (87.5% in this study and 85% in Fu et al. 1992) are heterozygous at this locus. The most frequent allele corresponds to five repeats—37% in our sample, versus >40% (Brook et al. 1992) and 35% (Mahadevan et al. 1992). We did not observe alleles between six and nine repeats, which is in agreement with the lack of alleles in this size range reported for a larger sample (Brook et al. 1992). Normal Mendelian inheritance was demonstrated for 12 meioses in non-DM branches of DM families (data not shown), which suggested no meiotic instability for the CTG fragments within the 5–35-repeat range. Our results in the French population, together with previous studies in other ethnic groups, corroborate the fact that the number of repeats probably does not exceed 35 in normal individuals.

CTG Repeat Length in DM Chromosomes

By analogy with the fragile X syndrome, we have tentatively defined three classes of mutations corresponding to three different phenotypes (classes M, A, and C). We thus analyzed DM patients belonging to the three different classes, in order to assess the possible relationship between the length of the CTG repeat and the severity of the disease. Southern blotting analysis was systematically performed with two restriction enzymes, *Eco*RI and *Bam*HI, and with probes cDNA25 and p5B1.4, respectively. The enlarged fragment migrating as an abnormal larger band was observed on *Eco*RI digests in two-thirds of the patients and/or on *Bam*HI experiments. The CTG expansion was thus ascertained in all 337 DM patients examined who belonged to the 116 families studied.

The distribution of length variation of the CTG region in DM patients belonging to the three different classes is shown in figure 2. The majority of the 77 class M patients had alleles with small insertions (mean size 440 bp). We included in this class 11 previously undiagnosed individuals who showed expansion of the CTG repeat in the 120–500-bp range. These subjects were suspected to be affected only on the basis of minor clinical signs and/or molecular family studies with closely linked polymorphic markers. The expansion in allele size was detected with *Bam*HI for most of the patients of class M, while *Eco*RI allowed us to detect the increase in patients of class A or class C. Patients of class A (213 individuals) showed, on average, larger insertions (mean size 1.9 kb), with size ranging from 200

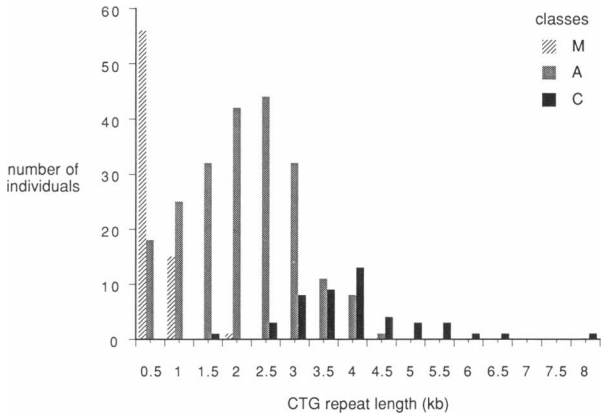

Figure 2 Distribution of size of expansion of the CTG repeat for the different classes of DM patients. The *X*-axis represents the CTG repeat length of the mutant gene, and the *Y*-axis represents the number of individuals observed with this repeat length. The total number of individuals examined was 337, including 77 minimally affected patients (class M), 213 patients presenting with the classic adult-onset type (class A), and 47 congenitally affected children (class C).

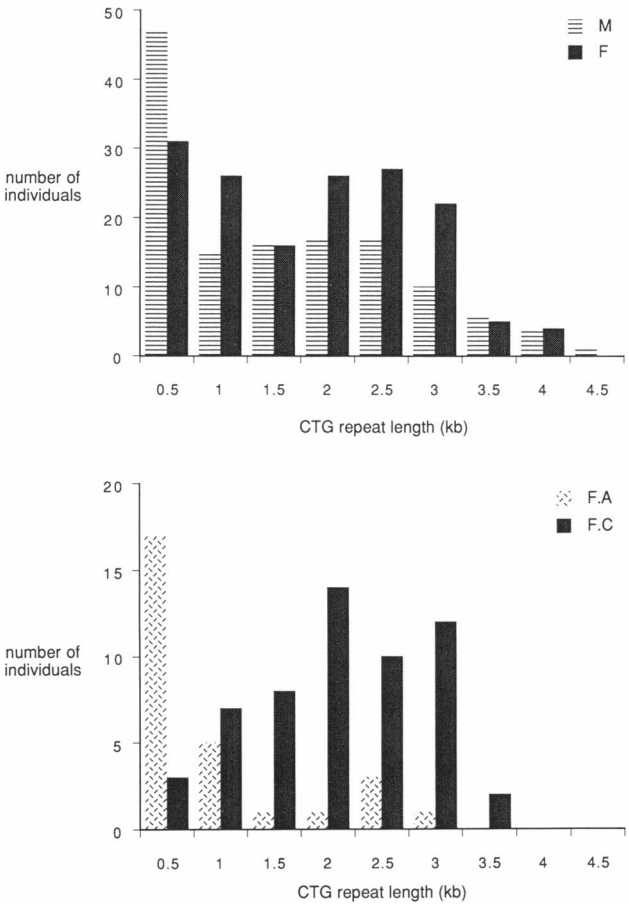

Figure 3 Comparison of CTG repeat length between 133 males (M) and 157 females (F) presenting with either the minimally affected type or the classic adult-onset form (*top panel*) and 23 mothers of adult-onset type (F.A) and 56 mothers of congenitally affected infants (F.C) (*bottom panel*). The *X*-axes represent the CTG repeat length of the mutant gene, and the *Y*-axes represent the number of individuals observed with this repeat length. The total number of chromosomes analyzed was 290.

bp to 4 kb. The largest alleles were observed in class C (47 children), with an expansion in the 1.5–8-kb range (mean size 3.8 kb).

The distributions of the CTG repeat length in the three different classes of patients—M, A, and C—were widely spread out and overlapped with each other. We thus confirm, on a large French population (337 patients), that, in spite of the correlation between the size of the repeat and the severity of the disease, the size by itself is not sufficient for accurate prediction of the severity of the disease and/or age at onset.

The top panel of figure 3 shows the distributions of the CTG repeat expansion in males and in females. In our DM sample, the mean size of the CTG region is slightly higher in females than in males: the sizes are 1.6 kb and 1.4 kb, respectively. Even though the range (from 120 bp to 4 kb) is similar in both sexes, 47% (62/133) of males exhibit elongation ≤1 kb, versus only 36% (57/157) of females. This could be due to a bias in sampling, since families with the most severe forms, often inherited from the mothers, are more likely to be recruited. Furthermore, women who have given birth to congenitally affected newborns have, on average, a larger CTG repeat (mean size 1.9 kb) than do mothers of patients with the adult-onset type (mean size 0.8 kb) (fig. 3, *bottom*). Of the former, 18% (10/56) had an expansion ≤1 kb, versus 82% (23/28) of the latter. Therefore, estimating the risk of having a congenitally affected child in a first pregnancy is hindered by the wide overlap between the two distributions.

Mitotic Instability

In white blood cells, smears of hybridization in Southern blot analysis, reflecting the mitotic instability, have been reported for the majority of DM patients with a CTG repeat length >1 kb, in males as well as in females (Mahadevan et al. 1992). In order to investigate whether this instability was limited to the larger (>1-kb) alleles and to lymphocytes, we compared the pattern of hybridization in lymphocytes of all patients and in different tissues from one DM fetus. As previously reported, a smear was often observed for patients of class A or class C. Furthermore, patients of class M, with expansion between 200 bp and 1.5 kb, also displayed somatic mosaicism observed on *Bam*HI digests (fig. 4). Mitotic instability is thus not limited to large alleles. It is thus more likely that this instability accounts for the presence of multiple PCR alleles in the same individual (Mahadevan et al. 1992); they are probably not artifacts of the PCR reaction (Brook et al. 1992).

Various tissues of a 20-wk-old fetus carrying the DM mutation (Lavedan et al. 1991) have been analyzed with p5B1.4/*Bam*HI. All eight tissues examined, including skeletal muscle, cardiac muscle, liver, lung, brain cortex, kidney, small bowel, skin, and bone, showed a normal hybridization signal at 1.4 kb and an abnormal enlarged band which varies from 3.0 kb to 3.4 kb (fig. 5). The lowest increase was seen in the liver, and the largest was seen in the kidney. In addition, this abnormal band had a blurred appearance, which reflects the size heterogeneity of the mutation. Therefore, our data suggest that there is mitotic instability in all fetal tissues, as observed in lymphocytes. As previously demonstrated

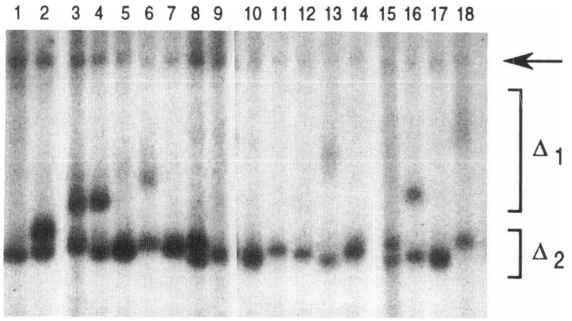

Figure 4 Mitotic instability in patients with a small CTG increase. Southern blot analysis (with *Bam*HI/p5B.1.4) revealed that all normal individuals not carrying the DM mutation (lanes 1, 2, 5, 7–12, 14, 15, and 17) exhibited alleles (Δ_2) close to 1.4 kb, corresponding to the presence of from 5 to about 30 CTG repeats. In contrast, all DM patients of class M (lanes 3, 4, 6, 13, 16, and 18) showed an abnormal large band (Δ_1) corresponding to the expansion of the CTG repeats. Some of these bands have a blurred appearance (lanes 6, 13, and 18), suggesting mitotic instability. Abnormal CTG amplification varies from 120 bp (lane 3) to 280 bp (lane 18). In lanes 2, 8, and 15, two distinct alleles are present in normal individuals. The arrow indicates the presence of a constant band.

for the fragile X syndrome (Devys et al. 1992), somatic heterogeneity of the mutation may be established during the very early stage of embryogenesis and/or depend on cell-specific mitotic instability. The identical pattern obtained with DNA extracted from a fresh blood sample or after Epstein-Barr virus transformation and in vitro culture (data not shown) does not support a relationship between the instability and the number of cell divisions, at least in these cells. Further studies of tissues from other DM patients, fetal or adult, male or female, should allow one to determine whether somatic mosaicism plays a role in the variable expression of the DM phenotype.

Predisposition to Congenital DM

In 47 sibships we observed that 42 (58%) of 72 children born after a child showing the neonatal form presented the same form of the disease (class C). In the remaining 30 (42%) there is, thus far, no evidence for the development of the adult-onset type of DM. Moreover, of the 30 healthy individuals, none of the 15 who were analyzed at the molecular level showed an expansion of the CTG repeat. It thus appears that, even though there is a wide variability of the size of the CTG sequence within a sibship, the affected sibs carry a mutation with an expansion leading to the same type of onset, adult (classes M and A) or congenital (class C). In addition, we noticed that transmitting sisters, whatever their clinical status or the repeat length, always gave birth to children affected with the same type of disease, either A or M (8 sisters from 4 families) or C (21 sisters from 10 families). For instance, in two pairs of sisters with very different repeat length—300 bp and 2,000 bp, versus 150 bp and 1,000 bp, respectively—the clinical status, as expected, correlated with the size of the repeat. Our observations thus suggest not only a maternal but also a familial predisposition. The "commitment" of sisters to give birth to children with the same form of the disease supports the hypothesis of a genetic factor, rather than a consequence of the expression of the maternal disease.

Dynamics of Intergenerational Expansion

To characterize the dynamics of the expansion, and to investigate a possible difference between male and female meioses, we studied the enlargement of the CTG region in 159 DM parent-child pairs. We compared the intergenerational variation between males (87 pairs) and females (72 pairs) and between mothers of patients of classes M and A (33 pairs) and mothers of patients of class C (39 pairs) (fig. 6).

The mean variation of the CTG repeat length is 1.8 kb (from −0.2 to +4.4 kb) after female meioses and is only 0.8 kb (from −2.6 to +3.6 kb) after male meioses (*t*-test; $P < .01$). This is in contrast to a previous study, by Tsilfidis et al. (1992), which reported no difference between 45 male and 42 female meioses. This may be explained, in part, by the fact that our sample is larger, including 159 parent-child pairs, and also by the fact that the mothers of congenitally affected children were

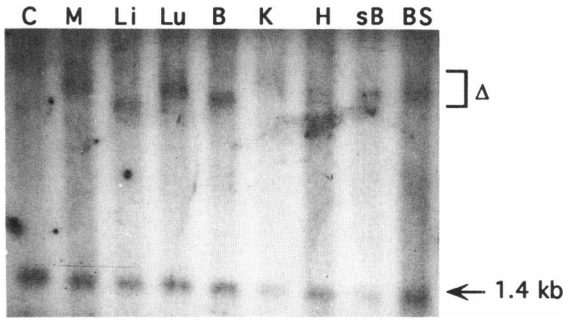

Figure 5 Somatic mosaicism in DM tissues, as shown in an autoradiogram of a *Bam*HI/p5B1.4 Southern blot experiment. The variation of the enlarged CTG expansion (Δ) in the different tissues of a 20-wk-old DM fetus is shown, above the normal 1.4-kb allele. Lane C, Non-DM control lymphocytes. Lane M, Skeletal muscle. Lane Li, Liver. Lane Lu, Lung. Lane B, Brain cortex. Lane K, Kidney. Lane H, Heart. Lane sB, Small bowel. Lane BS, Bone and skin.

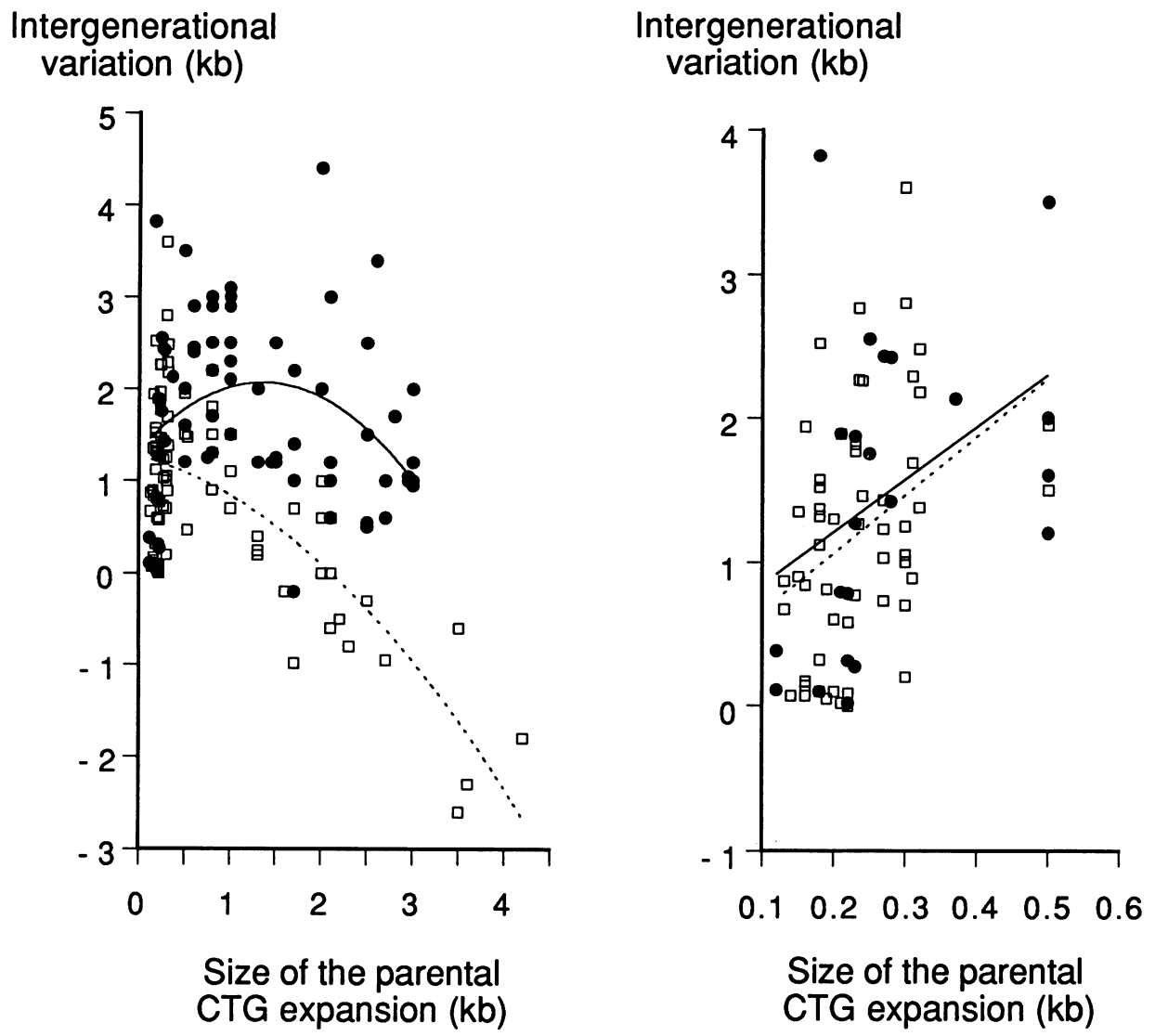

Figure 6 Intergenerational variation of the CTG sequence, after male and female meioses. The *X*-axis represents the CTG repeat length of the mutant gene of the affected parents, and the *Y*-axis represents the intergenerational variation of the parental CTG expansion calculated from the child CTG repeat length. Each dark dot and each white square correspond to one mother-child pair and to one father-child pair, respectively. Curves representing the overall distribution (*left panel*) or the linear regression in the 0–0.5-kb range (*right panel*) were determined by a computer program (Kaleidagraph): the broken line (- - -) is for father-child pairs, and the unbroken line (———) for mother-child pairs.

not included in the previous study. If we also exclude these women, the mean value for female meioses (1.5 kb) is still greater than that for male meioses (0.8 kb). In agreement with the data of Tsilfidis et al., birth of congenitally affected infants occurred after a more important enlargement (mean value 2.0 kb) of the mutation than for individuals of classes A and M, who inherited the disease from their mother (mean value 1.5 kb). The greater expansion after female meioses is observed whatever the size and clinical status of the mother and does not simply result from the larger repeat size in female parents, as shown in figure 6.

The instability of the CTG sequence appears to depend on its size as well as on the sex of the transmitting parent (fig. 6, *top*). Indeed, when the parental CTG expansion is in the 0–0.5-kb range, linear regression analysis (fig. 6, *bottom*) suggests that the importance of the enlargement depends on the number of repeats after

male as well as after female meioses: the correlation coefficient for female and male transmission is .40 and .37, respectively. This is thus similar to the relationship between the size of the fragile X premutation allele (from 156 bp to 348 bp) in the parent and that observed in the child (Fu et al. 1991). Beyond 0.5 kb, the intergenerational variation in DM was more important after female meioses (fig. 6, *top*). This might reflect a different mechanism in female and male meioses. Enlargement seems compulsory after female meioses, whatever the size of the maternal repeat (71/72 cases). In contrast, when the parental CTG expansion is ⩾1.5 kb, there is a tendency to compression almost exclusively after male meioses, in 78% (14/18 cases) (fig. 6, *top*). Two of the 12 affected fathers with a ⩾1.5-kb CTG repeat length transmitted the mutation to two of their children, with a decrease or no detectable enlargement of the CTG sequence. We also observed, for a father with a 1.7-kb CTG repeat sequence, a first affected child with a larger fragment (2.4 kb), while the second affected child had a smaller one (0.7 kb). This does not seem to be age related, since we did not detect any correlation between the paternal age and intergenerational variation of the number of CTG repeats. In male gametogenesis, selection against larger alleles or repair mechanisms leading to compression may be involved. Alternatively, these mechanisms could occur early in embryogenesis only when the mutation is paternally inherited.

Segregation Analysis

In the context of prenatal diagnosis, we noticed, in several families where congenital cases occurred, not only that the mother was slighly affected but also that it was difficult to determine which of the grandparents had transmitted the disease. Molecular analysis and further pedigree and clinical investigations revealed, in most cases, that the grandparent carrying the DM mutation was the grandfather. Figure 7 shows the result of the segregation analysis performed in 81 families, which confirms the existence of a significant excess of transmitting grandfathers. Among the 96 DM grandparent-child pairs studied, 65 involved transmitting grandfathers and only 31 involved transmitting grandmothers ($\chi^2 = 6.216$; $.01 < P < .02$). This excess was observed whenever the disease was inherited from the father or from the mother, in adult cases as well as in congenital cases.

We further analyzed the segregation of the disease in all our DM families, with particular interest in the sex ratio and in the fertility in males and females. The only

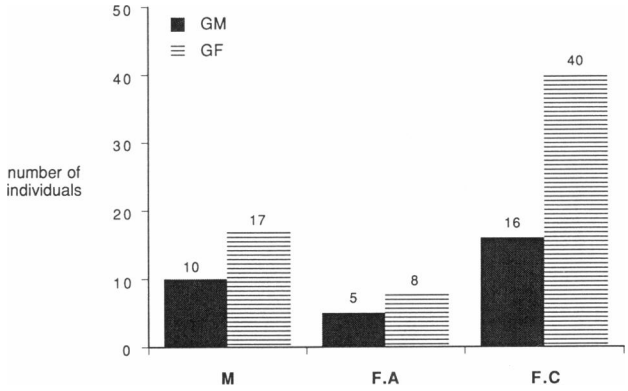

Figure 7 Grandparental origin of the DM mutation. The *Y*-axis represents the number of individuals observed in the different 96 grandparent-child pairs. In our sample, there are more transmitting grandfathers (GF) than transmitting grandmothers (GM), both for the entire sample—i.e., whatever the sex of the parent (M = male; and F.A and F.C = female)—and for females, whatever the form of the disease affecting the child (F.A = adult form; and F.C = congenital form).

significant difference was observed for the size of the sibships. We found that affected males have, on average, more children than do affected females. Although ascertainment bias cannot be rejected, the excess of grandfathers could be due to, at least, two biases: first, women with neonatal cases will not appear as grandmothers of affected patients, and, second, there are more children born to an affected male than to an affected female.

Conclusion

The observation of affected sisters having given birth to children affected in all cases with the same form of DM, either the adult or the congenital form, leads us to propose the existence of a familial predisposing effect. Since prognostic and diagnostic values of the CTG repeat length remain uncertain, it will be interesting to further investigate this hypothesis which may have major implications in genetic counseling.

Among the minimally affected individuals (class M), many individuals would have remained undiagnosed if they had not been related to more severely affected subjects. Since these individuals have a repeat length ranging from 120 bp to 500 bp (40 to 166 repeats), this range most likely represents the premutational state. The DM premutation range is therefore very close to that observed for fragile X syndrome—namely, 52 to 200 repeats (Fu et al. 1991). The largest allele size in normal individuals does not exceed 35 repeats, as re-

ported elsewhere (Brook et al. 1992; Davies et al. 1992; Fu et al. 1992; Mahadevan et al. 1992). Thus the insignificant difference between normal (35 repeats) and premutated individuals (40 repeats) suggests a continuum across the instability threshold. However, the complete linkage disequilibrium found between a 1-kb intragenic insertion polymorphism (Harley et al. 1991, 1992*a*; Mahadevan et al. 1992) and the DM mutation (Lavedan et al., submitted) suggests either a unique or a small number of ancestral DM mutations. If a similar disequilibrium can be found between this polymorphism (1-kb insertion) and the largest normal CTG alleles (≤35 repeats), then it could be postulated that normal individuals also carrying the 1-kb insertion are more prone to amplification toward the DM mutation. In fragile X syndrome the conversion from premutation to full mutation only occurs after female meiosis. This would apply to DM if the congenital form was considered as the only equivalent to the full-mutation fragile X phenotype, since this severe form of DM only appears following a female meiosis. This apparent analogy between fragile X and DM may not be integral and may depend on different mechanisms related to the different modes of transmission—X-linked for fragile X and autosomal (chromosome 19) for DM.

Morton and Macpherson (1992) recently proposed a four-allele model for fragile X: allele N for normal CGG repeats, alleles S and Z for small inserts, and allele L for full mutations. Since the conversion from Z to L only occurs through female meiosis, this model could also apply to DM. Under this hypothesis the adult form of DM would correspond to the Z allele (Morton and Macpherson 1992). On the basis of our data it should be stressed that compression, corresponding to the reversion from Z to S, occurs almost exclusively in male meioses. However, similar conversion probabilities in the two sexes are observed in the conversion from N to S, with a positive correlation between the size (≤0.5 kb) of the parental mutated allele and the intergenerational variation in fathers as well as in mothers.

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