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Maternal Mosaicism for a Second Mutational Event in a Type I Spinal Muscular Atrophy Family

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Summary

Spinal muscular atrophy (SMA) is a common fatal motor-neuron disorder characterized by degeneration of the anterior horn cells of the spinal cord, which results in proximal muscle weakness. Three forms of the disease, exhibiting differing phenotypic severity, map to chromosome 5q13 in a region of unusually high genomic variability. The SMA-determining gene (SMN) is deleted or rearranged in patients with SMA of all levels of severity. A high de novo mutation rate has been estimated for SMA, based on the deletion of multicopy microsatellite markers. We present a type I SMA family in which a mutant SMA chromosome has undergone a second mutation event. Both the occurrence of three affected siblings harboring this same mutation in one generation of this family and the obligate-carrier status of their mother indicate the existence of maternal germ-line mosaicism for cells carrying the second mutation. The existence of secondary mutational events and of germ-line mosaicism has implications for the counseling of SMA families undergoing prenatal genetic analysis.

Introduction

Childhood-onset proximal spinal muscular atrophy (SMA) (MIM 253300, 253550, and 253400) is a common neurodegenerative disorder affecting ~1 in 10,000 live births per year (Dubowitz 1995). The disease is classified, on the basis of age at onset and clinical severity, into three subtypes: type I (severe), type II (intermediate), and type III (mild) (Munsat and Davies 1992). All three forms of SMA map to one locus on chromosome 5q13 (Brzustowicz et al. 1990; Gilliam et al. 1990; Melki et al. 1990*a*, 1990*b*). The SMA-determining gene, the survival motor-neuron gene (SMN), is present in two almost identical copies, each of which is contained within a large inverted duplicated region (Lefebvre et al. 1995). The telomeric SMN gene (SMNtel) is deleted or rearranged in patients with SMA of all levels of severity (Bussaglia et al. 1995; Cobben et al. 1995; Hahnen et al. 1995, 1997; Lefebvre et al. 1995; Rodrigues et al. 1995, 1996; Brahe et al. 1996; Matthijs et al. 1996; Parsons et al. 1996; Velasco et al. 1996; Simard et al. 1997; Talbot et al. 1997*a*). Gene conversion was implicated in the disease mechanism when patients in whom SMNtel exon 7 was deleted but SMNtel exon 8 was retained were found to contain hybrid genes with SMNcen exon 7 linked to SMNtel exon 8 (Bussaglia et al. 1995; Cobben et al. 1995; Hahnen et al. 1995, 1996; Lefebvre et al. 1995; Rodrigues et al. 1995, 1996; Devriendt et al. 1996; van der Steege et al. 1996; Velasco et al. 1996; DiDonato et al. 1997; Simard et al. 1997; Talbot et al. 1997*b*). Direct physical evidence for the role of gene conversion of the entire SMNtel gene, including exon 8, in SMA types II and III was obtained from pulsed-field genomic analysis of the SMA region (Campbell et al. 1997). The clinical phenotype appears to be determined by the underlying mutational mechanism. Most cases of severe SMA result from gene-deletion events, whereas the milder forms of the disease result from gene conversion of SMNtel to its centromeric counterpart, SMNcen. Differences in SMA phenotype may therefore be accounted for by the number of SMNcen gene copies present. Patients suffering from the milder forms of SMA contain more SMNcen copies, as a result of gene conversion of SMNtel, each of which produces a small amount of functional full-length SMN protein (Campbell et al. 1997; Coovert et al. 1997; Lefebvre et al. 1997). Recent studies involving the identification of protein-protein interactions by yeast two-hybrid screening have suggested possible roles for the SMN protein product both in spliceosomal small nuclear ribonucleoprotein biogenesis and in the prevention of

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apoptotic cell death via interaction with bcl-2 (Fischer et al. 1997; Iwahashi et al. 1997; Liu et al. 1997).

Each duplicated region also contains either a functional or a pseudogene copy of the gene encoding the neuronal apoptosis-inhibitory protein (NAIP) (Roy et al. 1995). Deletions of the functional telomeric NAIP gene are most often associated with type I SMA (Hahnen et al. 1995; Roy et al. 1995; Wirth et al. 1995; Burlet et al. 1996; Rodrigues et al. 1996; Velasco et al. 1996).

A third gene, BTF2p44, encoding a subunit of the transcription factor TFIIH, exists in each duplicated region (Humbert et al. 1994; van der Steege et al. 1995*a*). A correlation has been shown between deletion of the telomeric BTF2p44 gene and severe SMA, but the protein structure and function were found to be normal in patients homozygously deleted for this gene, which suggests that it is not critical in the development of SMA (Burglen et al. 1997).

The genomic region containing the gene mutated in SMA is highly variable in size among unrelated individuals, even in a highly homogeneous population from northern Finland (Campbell et al. 1997). Chromosome 5–specific repeat sequences are known to exist in the region. Pseudogene sequences of the β-glucuronidase and Br-cadherin genes have been shown to account for some of these and, by mediation of recombination events, may contribute to instability (Sargent et al. 1994; Theodosiou et al. 1994; Selig et al. 1995). In addition, sequences homologous to the human retrotransposable element, THE1, may be implicated in the generation of instability in the SMA region (Francis et al. 1995). THE1 elements are also known to occur in the dystrophin gene, in regions with a particularly high frequency of deletion (Pizzuti et al. 1992; McNaughton et al. 1993).

The inherent instability of the SMA region is further demonstrated by the occurrence of de novo deletions involving the multicopy microsatellite markers C272 (or Ag1CA) and C212, which are associated with each SMN gene copy (Melki et al. 1994; Capon et al. 1995; Daniels et al. 1995). The identification of a de novo deletion involving both of these markers in the more severely affected of two haploidentical siblings, both of whom showed SMNtel deletion, highlights the unstable nature of this region (Parano et al. 1996). The identification of seven de novo mutations involving loss of these markers in a study of 340 families has led to an estimation of the mutation rate for SMA as 1.1×10^{-4}, suggesting a high rate of mutation in this region (Wirth et al. 1997). The same study determined that, in six cases, the mutation was paternal in origin, implicating rearrangement during paternal meiosis as the major mutational mechanism.

Here we present an unusual family in which type I SMA appears to result from a secondary mutational event on a chromosome already carrying an SMA mutation. This result suggests that, in addition to a high de novo mutation rate, some SMA mutant chromosomes may harbor a propensity to undergo further mutation. Both the occurrence of the disease in three haploidentical siblings and the pulsed field–gel electrophoresis (PFGE) analysis of this family indicate the existence of maternal germ-line mosaicism for this mutation. The analysis of such a family by PFGE presents a unique opportunity to follow the progress of such a mutational event and to determine its origin. The occurrence of germ-line mosaicism has implications for genetic counseling.

Subjects, Material, and Methods

Subjects

All patients conformed to internationally agreed diagnostic criteria (Munsat and Davies 1992). Informed consent was obtained from all subjects. DNA for haplotype analysis was prepared from blood taken into Na-EDTA by the Nucleon II method (Anachem).

PCR Analysis

Genotyping of DNA from family members was carried out by standard PCR analysis incorporating radiolabeled α-[^{35}S] d-ATP, as described elsewhere (Daniels et al. 1992; Morrison et al. 1992). Genotypes were obtained at the following loci: D5S679, D5S125, D5S681, D5S435, D5S557, D5S610, D5S112, and D5S39CA. Analysis with CATT-alt (a sublocus of the multicopy marker C161) primers was carried out as described by Daniels et al. (1995).

PFGE

High–molecular-weight genomic DNA was prepared from cultured lymphocytes embedded in low–gelling-temperature agarose and was subjected to PFGE, as described elsewhere (Campbell et al. 1997).

Results

Family Genotypes in the SMA Region

Genotyping of the family whose pedigree is shown in figure 1 was carried out with microsatellite markers flanking the SMA locus on chromosome 5q13. Genotype results are shown diagrammatically as individual chromosomes inherited by each member of the family. The three affected siblings, III_1, III_2, and III_3, were haploidentical at all loci analyzed. The carrier status of unaffected siblings was determined by this haplotype analysis. Genotyping of this family with primers flanking CATT-alt revealed the passage of null alleles from both grandparental carrier individuals to each carrier parent (fig. 2) (Melki et al. 1994; Daniels et al. 1995). Null

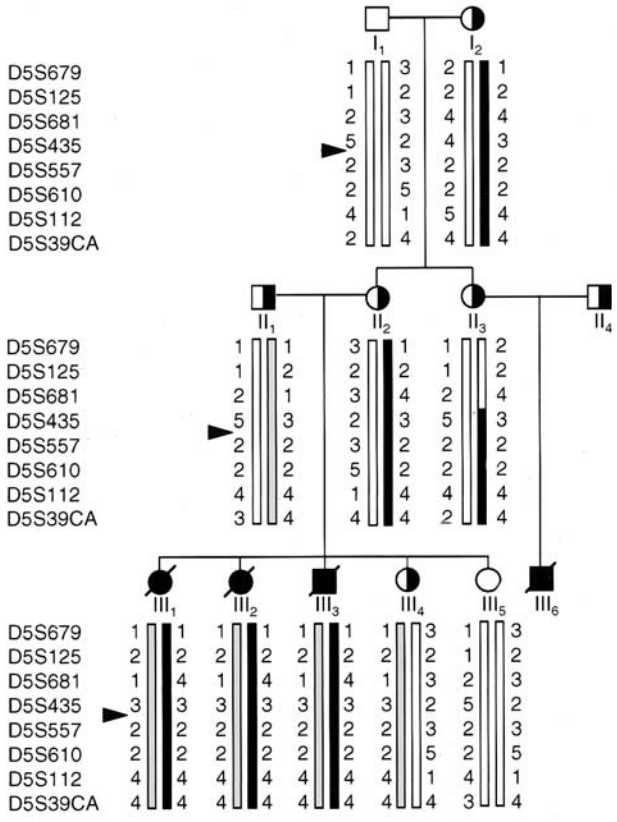

Figure 1 Genotype analysis of three generations of a type I SMA family. PCR amplification involving incorporation of α-[^{35}S] d-ATP was carried out by means of primers flanking microsatellite repeats surrounding the SMA locus on chromosome 5q13. Markers used are as shown (*left*). The position of the SMA locus relative to the two closest flanking markers used is shown by an arrowhead. Black bars represent the maternal and grandmaternal mutant chromosome 5. The paternal mutant chromosome 5 is shaded gray. Individual II_3 has inherited a recombination event that occurred proximal to the SMA locus and that does not affect her inheritance of the mutant SMA gene. DNA from individuals II_4 and III_6 was unavailable for analysis. All three affected siblings, III_1, III_2, and III_3, were haploidentical at all loci tested. Individuals in generation III are not depicted according to convention, with the eldest on the left, but instead are shown in the order in which they appear on the pulsed-field gel shown in figure 4, for ease of comparison.

alleles of this marker were reported, elsewhere, to be in linkage disequilibrium with the disease in a Finnish population, suggesting close association of this marker with the disease gene (Daniels et al. 1995). All three affected siblings showed homozygous inheritance of null alleles at this locus. The obligate-carrier status of individual II_2 was suggested not only because three of her offspring were affected by SMA type I but also because her sister, individual II_3, inherited the same mutant chromosome from their mother and also had a child (individual III_6) affected by SMA. The passage of a null allele at the CATT-alt locus by her carrier mother is further evidence of the carrier status of individual II_2.

Identification of Maternal Germ-Line Mosaicism

Several members of this SMA type I family were analyzed by PFGE, as part of a larger study, to investigate further the molecular basis of the SMA mutation. In such analysis, genomic DNA is subjected to sequential hybridization with probes corresponding to exons 3 (SMN3) and 8 (SMN8) of the SMN gene and to exon 5 of the NAIP gene (NAIP5). (For detailed methodology, see Campbell et al. 1997.) Figure 3 shows a schematic representation of the positions of the probes used, in relation to the position of genes in the SMA region. Probes SMN3 and SMN8 detect all SMN copies, whether centromeric or telomeric in origin. In those patients in whom deletion of SMNtel exons 7 and 8 has occurred, only SMNcen copies will hybridize to SMN8. In patients showing no PCR amplification of SMNtel exons 7 and 8 but in whom gene conversion of SMNtel to SMNcen has occurred, SMN8 will still hybridize to

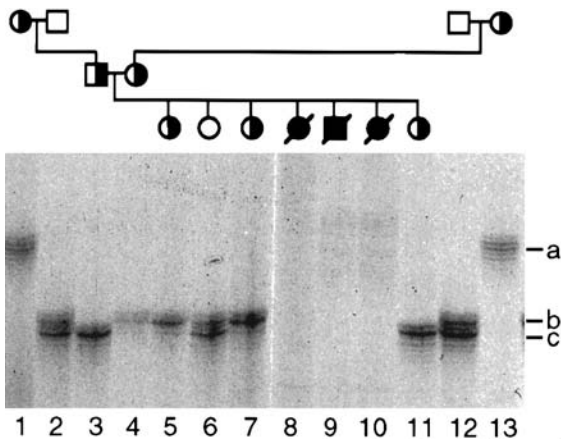

Figure 2 Inheritance of null alleles in the SMA region. DNA from family members was analyzed with primers flanking a sublocus of the multicopy marker C161, termed "CATT-alt." Analysis was performed on those individuals whose genotypes are shown in figure 1, as well as on some additional family members. Here, individuals are shown according to convention: those in lanes 5 and 6 represent individuals III_4 and III_5, respectively, both of whom have inherited the paternal mutant chromosome. The youngest carrier child inherited the maternal mutant chromosome, but this child died of causes unrelated to SMA and could not be included in the PFGE analysis shown in figure 4. Alleles a, b, and c are indicated (*right*). This figure shows the inheritance of null alleles, or lack of PCR amplification products, in carrier and affected individuals. The inheritance of null alleles can be traced back to the grandparental generation on both sides of the family. For example, allele a was not inherited by the father of the affected children (lane 3) from his mother (lane 1). In addition, allele a was not inherited by the mother (lane 4) from her mother (lane 13). Both null alleles were therefore passed, on each parental mutant chromosome, to the affected children, who are homozygous null for this allele.

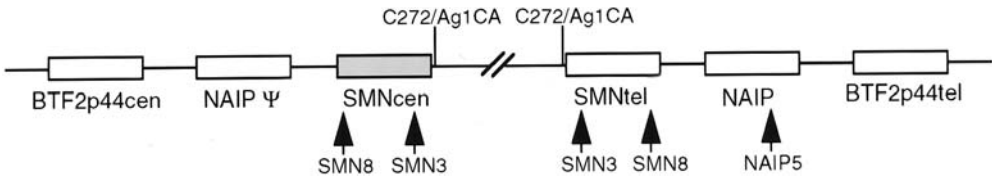

Figure 3 Schematic representation of the SMA region on chromosome 5q13. The inverted duplication containing the SMN, NAIP, and BTF2p44 genes is shown diagrammatically, with the region depicted by the broken line representing an unknown physical distance. The region is not drawn to scale. The fully functional SMNtel gene is shown as an unblackened box, the partly functional SMNcen gene as a gray box. The NAIP genes and the BTF2p44 genes are as labeled. NAIPy represents the centromeric pseudogene copy of NAIP, lacking exon 5. The approximate positions of the exon-specific probes used in PFGE analysis are shown by arrows. The approximate position of the multicopy marker C272 (or Ag1CA) is indicated at the 5′ end of each SMN gene copy.

all SMN gene copies. Probe NAIP5 detects only those DNA fragments originating from the telomeric duplicated region, since the functional telomeric NAIP gene contains exon 5, whereas the centromeric pseudogene copy does not (Roy et al. 1995). Thus, DNA fragments corresponding to SMNtel and SMNcen gene copies can be distinguished. However, in the case of gene conversion of SMNtel to SMNcen, the resulting DNA fragment will hybridize to NAIP5 by virtue of its physical location within the fragment, even though the fragment no longer contains the SMNtel gene sequence.

The results of PFGE analysis are presented in figure 4. *Eag*I-digested genomic DNA was hybridized sequentially with SMN3 (fig. 4*A*), NAIP5 (fig. 4*B*), and SMN8 (fig. 4*C*). PFGE analysis yielded an unusual observation in the mother of this family (individual II_2). Although this individual is an obligate carrier for SMA, panel *B* of figure 4 indicates the presence of two DNA fragments hybridizing to NAIP5, which suggests the presence of two copies of SMNtel. Approximately 2% of carrier individuals possess two SMNtel gene copies, both existing on one chromosome (McAndrew et al. 1997). However, analysis of the two unaffected children in this family, individuals III_4 and III_5, shows that this is not the case in individual II_2. The same single copy of NAIP5 was inherited by both unaffected individuals (fig. 4*B*, lanes 6 and 7). The two maternal SMNtel gene copies must therefore segregate independently. No maternal SMNcen copy was inherited by child III_5, whose DNA is shown in lane 7. The maternal SMNcen copy (the smallest hybridizing band in lane 2) is therefore expected to segregate with the NAIP5 hybridizing fragment not inherited by these two unaffected offspring. This chromosome does not appear to have been inherited by any of the offspring in this family. Haplotype analysis revealed that one unaffected child (III_4) inherited a mutant SMA chromosome from her father and a normal chromosome 5 from her mother, whereas the other unaffected child (III_5) inherited the normal chromosome from each parent (see fig. 1). The maternal mutant chromosome must therefore be that which appears to carry one SMNcen gene and one SMNtel gene. This chromosome would be considered normal with respect to SMA, a suggestion that conflicts with the obligate-carrier status of individual II_2. The observation of a chromosome that is expected, on the basis of haplotype analysis, to carry a mutant SMA allele but that appears to carry an SMNtel gene in addition to an SMNcen gene may be explained by gene conversion of the SMNtel copy to SMNcen. This would result in a mutant chromosome with no SMNtel copy and two SMNcen copies. A schematic representation of the chromosome complement of SMN genes in individual II_2 is presented in figure 4*D*. The PFGE results are also summarized and presented as SMN and NAIP genotypes in figure 5. The maternal mutant chromosome representing what is termed here the "primary mutation" (i.e., gene conversion of SMNtel to SMNcen) is shown as haplotype C′-D-D in figure 5.

The three affected siblings appear to have inherited only one SMN gene copy, which does not cohybridize with NAIP5 and which is therefore is considered to be centromeric. This is consistent with the severe clinical course observed in these patients and with the PCR deletion analysis that showed lack of amplification of SMNtel exons 7 and 8, as well as lack of amplification of NAIP exon 5 (data not shown; Lefebvre et al. 1995; Roy et al. 1995; van der Steege et al. 1995*b*). Determination of the parental origin of the SMNcen copy inherited by these affected individuals is complicated by the existence of an SMNcen band of similar size in each parent (figs. 4*A* and *C*, lanes 1 and 2 [the smallest hybridizing band in each case]). As described above, the maternal SMNcen copy, represented by the lowest SMN-hybridizing band in lane 2 of figure 4, is expected to cosegregate with the highest of the two NAIP5-hybridizing fragments in lane 2 of figure 4. This is clearly not the case in the three affected siblings, and it is assumed, therefore, that the SMNcen copy in these individuals is paternal in origin. The possibility exists that the single SMN-hybridizing *Eag*I fragment in each affected child

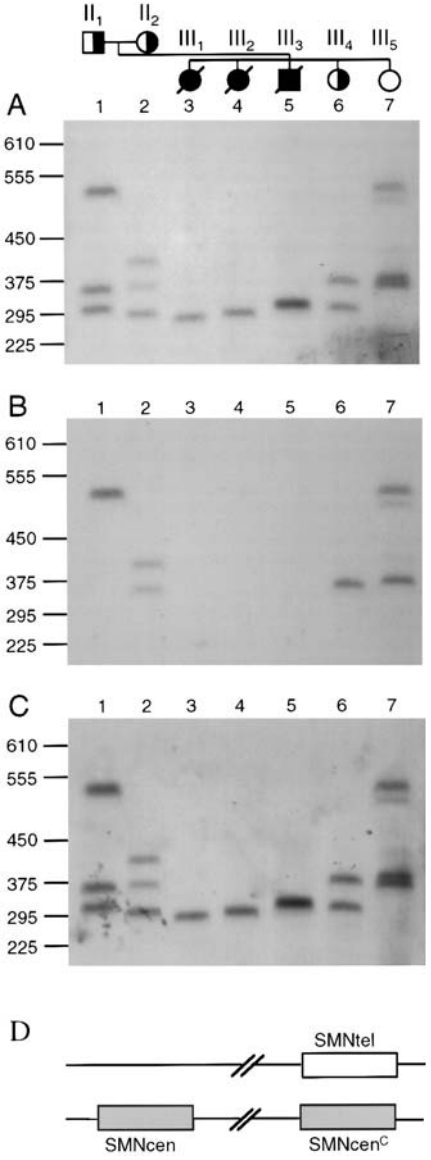

Figure 4 PFGE analysis of a type I family showing maternal germ-line mosaicism for a second SMA mutation. Lanes 1–7 contain genomic DNA from family members, digested with *Eag*I. Individuals II_1–III_5 are those individuals labeled thus in figure 1. Hybridizations were carried out by means of probes SMN3 (*A*), NAIP5 (*B*), and SMN8 (*C*). Washes were carried out in 0.5 × SSC/0.1% SDS at 65°C. Membranes were stripped between hybridizations, and the lack of signal was determined by autoradiography. PFGE was carried out at 170 V for 40 h, with pulse-switch time 60 s. Sizes (in kb) relate to yeast chromosome pulsed field gel (PFG) markers. The carrier status of unaffected siblings was determined by haplotype analysis (see fig. 1). A schematic representation of the likely SMN genotype of the mother (individual II_2) is presented in panel *D*. The upper line represents the normal maternal chromosome 5 containing one SMNtel gene only. The lower line represents the mutant maternal chromosome 5 containing two SMNcen copies, one of which ($SMNcen^C$) has arisen by gene conversion and therefore exists within the telomeric duplicated region.

contains two SMNcen gene copies, one inherited from each parent, although this is considered unlikely (see Discussion).

Discussion

The PFGE analysis of this SMA type I family revealed an unusual result in the carrier mother of three haploidentical affected individuals. Haplotype analysis showed that the mutant SMA chromosome in the mother (II_2) of these affected children was also inherited by her sister (II_3), who also had a child with SMA, lending weight to the carrier status of individual II_2. It is likely that the mutant chromosome 5 carried by individual II_2 contains two SMNcen gene copies, one of which has arisen by gene conversion from SMNtel. The gene-conversion event that is assumed to have given rise to this mutant chromosome did not involve the functional NAIP gene, and it would therefore be expected that the sister's affected child (individual III_6) would show amplification and hybridization of NAIP exon 5, unless a similar second mutation event had occurred in this side of the family. DNA from this child is unavailable, and the child has since died. Although the disease in this child could have occurred as the result of a new mutation, this seems highly unlikely in view of (*a*) the segregation of SMA in both sides of the family and (*b*) the inheritance, by both sisters, of a null CATT-alt allele from their carrier mother.

Whichever gene complement is present on the mutant maternal chromosome, none of the affected children possess hybridizing bands indicative of having inherited this chromosome. All three possess only one SMNcen copy, inherited from their father. They do not appear to have inherited any SMN gene copies from their mother. The SMN and NAIP genotypes in this family, as determined by direct analysis of the region by means of gene-specific probes, are summarized in figure 5. The observations made in this family can be explained by maternal germ-line mosaicism resulting from a second mutational event in a maternal chromosome already carrying a mutant gene-converted SMA allele, (i.e., two copies of SMNcen; haplotype C′-D-D in fig. 5). For example, a chromosome 5 deletion occurring in a germ cell of individual II_2 may have resulted in a population of mutant germ cells that gave rise to the three affected siblings in this family. Such a deletion event could result in the loss of all SMN and NAIP genes on the already mutant chromosome and is represented by maternal haplotype N-N-N in figure 5. Affected offspring would therefore appear to inherit no SMN or NAIP gene sequences from their mother—in other words, null alleles of these genes—as shown in figure 4.

A second possibility exists, in which a smaller deletion

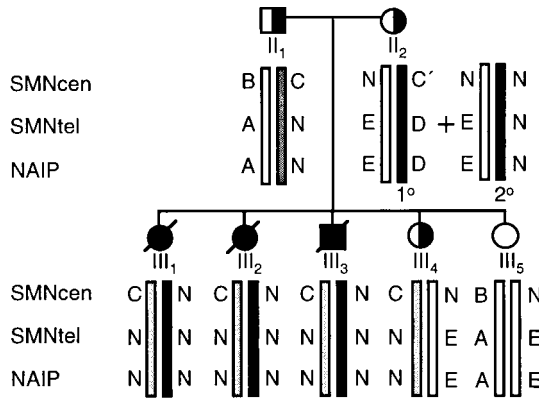

Figure 5 Representation of SMN and NAIP genotypes as determined by the pulsed-field genomic analysis shown in fig. 4. Alleles shown here correlate with the *Eag*I fragments shown in figure 4*A*, as follows: A = highest band, lane 1; B = middle band, lane 1; C = lowest band, lane 1; D = highest band, lane 2; E = middle band, lane 2; and C′ = lowest band, lane 2. Alleles C and C′ are so labeled since they are similar in size and their inheritance cannot be accurately followed in the affected offspring. Alleles B and E also appear similar in size in figure 4*A* but can be distinguished by hybridization with NAIP5. Null alleles, indicative of gene-deletion events, are represented by "N." Normal chromosomes 5 are shown as unblackened bars, mutant chromosomes as gray-shaded (paternal) or blackened (maternal) bars. The maternal chromosomes 5 carrying the proposed primary and secondary mutation events are designated "1°" and "2°," respectively.

event has occurred, allowing the original (not gene-converted) SMNcen gene copy to be retained on the maternal mutant chromosome. In such circumstances, the three affected siblings would be expected to carry a maternal SMNcen copy in addition to the SMNcen copy inherited paternally. The maternal chromosomal contribution, in such a scenario, would be represented by the haplotype C′-N-N, according to the nomenclature used in figure 5. As described earlier, one SMNcen copy in each parent appears almost identical in size (see figures 4*A* and *C*, lanes 1 and 2 [the smallest hybridizing band in each case]). It is possible that a hybridizing band of this size could contain two SMN gene copies, one inherited from each parent. However, there does not appear to be increased dosage in the lanes in figure 4 that contain DNA from the affected individuals, compared with other lanes; the band in lane 5 does appear slightly more intense, but not enough to account for two gene copies. The slight discrepancy in size between the same fragment in all three affected siblings results from differences in the amount of DNA in the agarose blocks loaded in each lane. The observation that DNA in lane 5 has run slightly more slowly equates to the presence of more DNA in this lane, explaining the slight increase in band intensity in this individual. A similar difference in fragment size in the three siblings was observed after hybridization of the same filter with a probe elsewhere in the region, which hybridized to fragments different from those hybridizing to SMN3 and SMN8 (data not shown). It therefore seems likely that the SMN-hybridizing fragment in each of the three affected siblings harbors only one paternally inherited SMNcen gene copy and that no maternal DNA corresponding to this region of chromosome 5 has been inherited. The SMN/NAIP genotypes in the affected offspring (individuals III_1, III_2, and III_3) are therefore presented as paternal contribution C-N-N and maternal contribution N-N-N in figure 5.

In an attempt to clarify this issue, analysis of this family was undertaken with the multicopy marker C272 (Melki et al. 1994). The hope was that, by determination of the C272 haplotype on each parental mutant chromosome, the genotype of the three affected siblings could be resolved. Since each SMN gene copy is closely associated with one C272 allele, it should then have been possible to determine the number of SMN gene copies present in the single PFGE fragment observed in each affected child. However, the same C272 allele was found to segregate with both the maternal and the paternal mutant chromosome (data not shown). Thus, only one C272 allele was observed in each affected child, but it could not be determined whether these individuals were homozygous or hemizygous for this allele.

A third, less likely, possibility is somatic mosaicism in individual II_2. In this case, N-N-N may be the original mutant genotype, and C′-D-D may have arisen by gene conversion. The paternally inherited genotype N-E-E might serve as a template for gene conversion of N-N-N to C′-D-D, a genotype that may or may not represent an SMA mutation.

The observation of this mutational event in a type I SMA family highlights the inherent instability of the SMA region and the high mutation rate in this region. The use of PFGE enabled us to determine the nature of the mutation as a secondary event on an already mutant chromosome, by highlighting the existence of two telomeric gene regions in the chromosomes of the mother of the family, indicating the presence of a gene-converted allele. The combined use of haplotype analysis and PFGE allowed us to propose maternal germ-line mosaicism as the underlying mutational mechanism in this case. It is interesting to note that the severity of the disease in the affected individuals of this family was likely to have increased as a result of the second mutation, in this case a deletion event. The prevalence of gene conversion in SMA type I has not yet been determined, since no type I patients retaining the NAIP gene were available for analysis by PFGE in the original study (Campbell et al. 1997).

The data presented here have implications for the counseling of families. The SMA phenotype may evolve, within a family, as a result of second mutational events

such as that described here. Alternatively, where there is a known new mutation, the risk of further affected children may be higher than the de novo mutation rate alone.

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Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for spinal muscular atrophy types I, II, and III [MIM 253300, 253550, and 253400, respectively])

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