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Frameshift mutation in the survival motor neuron gene in a severe case of SMA type I

Christina Brahe*, Olivier Clermont[1], Stefania Zappata, Francesco Tiziano, Judith Melki[1] and Giovanni Neri

Istituto di Genetica Medica, Università Cattolica del Sacro Cuore, Facoltà di Medicina e Chirurgia 'A.Gemelli', I-00168 Rome, Italy and [1]Unité de Recherches sur les Handicaps Génétiques de l'Enfant, INSERM U-393, Hôpital des Enfants Malades, 75743 Paris, France

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Recently, a spinal muscular atrophy (SMA) determining gene, termed survival motor neuron (SMN) gene, has been isolated from the 5q13 region and found deleted in most patients. A highly homologous copy of this gene has also been isolated and located in a centromeric position. We have analyzed 158 patients (SMA types I–IV) and found deletions of SMN exon 7 in 96.8%. Mutations other than gross deletions seem to be extremely rare. In one of the undeleted SMA type I patients, a newborn who survived for only 42 days, we detected a maternally inherited 5 bp microdeletion in exon 3, resulting in a premature stop codon. By RT–PCR and long range PCR amplification we were able to show that the deletion belongs to the SMN gene, rather than to the centromeric copy, and that the proposita had no paternal SMN gene. Analysis of the neuronal apoptosis inhibitor protein (NAIP) gene, which maps close to SMN and has been proposed as a SMA modifying gene, suggests the presence of at least one full-length copy. Haplotype analysis of closely linked polymorphic markers suggests that the proposita also lacks the maternally derived copy of the centromeric homologue of SMN supporting the hypothesis that the severity of the phenotype might depend on the reduced number of centromeric genes in addition to the frameshift mutation.

INTRODUCTION

Proximal spinal muscular atrophies (SMA) are a group of motor neuron diseases characterized by degeneration of spinal cord anterior horn cells and muscular atrophy. On the basis of age of onset and severity of the clinical course, three forms of childhood-onset SMA (types I, II and III) (1), and one adult-onset form (type IV) (2) can be distinguished. Childhood-onset SMA is one of the most frequent autosomal recessive diseases and a leading cause of infant mortality. Type I (Werdnig–Hoffmann disease) is the most severe form with onset within the first 6 months of life. The children are never able to sit without support and usually die within 2 years. Type II (intermediate form) has onset before the age of 18 months. Children are unable to stand or walk and death usually occurs after the age of 2 years. Type III (Kugelberg–Welander disease) is a mild, chronic form with onset after the age of 18 months.

An SMA determining gene, termed survival motor neuron (SMN) gene, has recently been isolated from the 5q13 region (3). A highly homologous copy of this gene has also been isolated and located in a centromeric position (3). SMN differs from its centromeric copy at only five nucleotides. Three of these differences occur in introns and two in exons; these latter are a synonymous mutation in exon 7 and a change in the 3′ non coding region of exon 8, respectively (3). These latter two base substitutions allow us to distinguish SMN exons 7 and 8 from the corresponding exons of the centromeric counterpart by single strand conformation polymorphism (SSCP) analysis or by restriction enzyme digestion of PCR amplification products (4). Molecular studies have shown that the vast majority of SMA patients are homozygously deleted for SMN exons 7 and 8 (3,6–8) independent of the severity of the disease, ranging from the most severe form with onset in the perinatal period to the adult form with onset in the 3rd–4th decade (9–10). The precise extent of these deletions is unknown, but patients affected with the severe form frequently have larger deletions than those with SMA types II and III (11,12) including the adjacent marker loci C212 and C272 (13) (Ag1-CA; 14) and/or the closely located gene encoding the neuronal apoptosis inhibitor protein (NAIP) (15).

Mutations other than gross deletions seem to be extremely rare. One point mutation and two microdeletions in splice junctions were detected in three patients (3). Recently, a frameshift mutation in exon 3 of SMN has been identified in four unrelated Spanish patients (16). We report here on a 5 bp microdeletion in exon 3 in a very severely affected Italian infant.

*To whom correspondence should be addressed

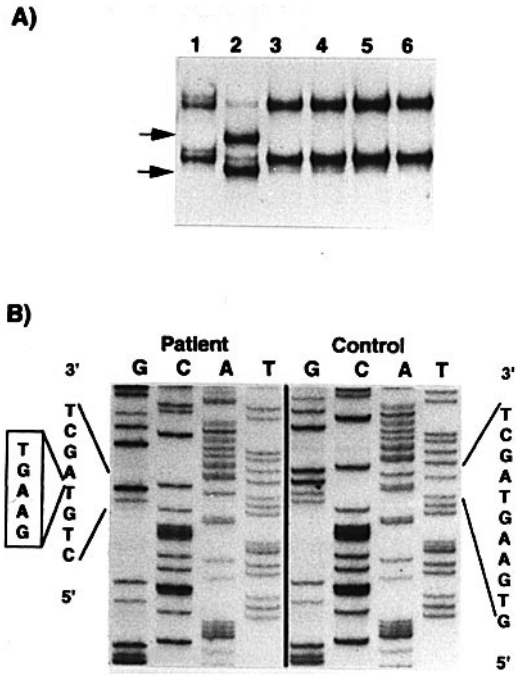

Figure 1. (**A**) SSCP analysis of exon 3. Lane 1, control individual; lane 2, proband; lanes 3–6, SMA patients. Arrows indicate band shifts in the exon of the proband. The other patients have a normal exon 3. (**B**) Sequence analysis of exon 3 from the patient and a control individual showing the 5 bp deletion in the proband.

RESULTS

Identification and characterization of the frameshift mutation

We have performed deletion analysis of exons 7 and 8 of the SMN gene in 158 unrelated patients (78 SMA type I, 36 type II, 39 type III and five type IV), diagnosed following the strict criteria of Munsat (17). By using both SSCP analysis (3) and restriction enzyme digestion of PCR amplification products (4) we found homozygous deletions of at least SMN exon 7 in 153 patients (96.8%).

The DNAs of the five patients who were not deleted for exon 7 (two type I, one type II and two type III) were studied for the presence of mutations in SMN exons 1–6 by SSCP analysis. An abnormal SSCP pattern was found in exon 3 of one individual (Fig. 1A), a girl, with a clinical and electrophysiological diagnosis of SMA type I. She had difficulties with feeding, severe hypotonia and muscle weakness since birth. Her mother, a physician, had not noticed reduction of fetal movements compared with her previous pregnancy. The parents are normal and non related and a normal brother is healthy. Although she did not have a muscle biopsy all her clinical parameters were typical of SMA I. The newborn died of respiratory failure at 42 days.

SSCP analysis of the parents revealed the same pattern of migration of the mother's exon 3. Sequencing of the PCR amplified exon 3 of the proposita showed a 5 bp deletion at codons 147–148 resulting in a premature stop codon, four nucleotides downstream (Fig. 1B).

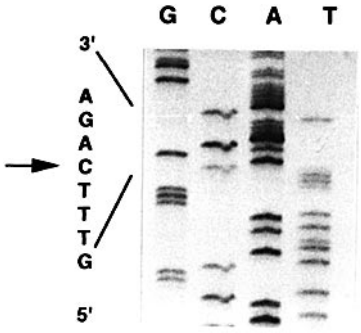

Figure 2. Sequence anlaysis of exon 7 of the mother's cDNA produced by amplification with the specific primer corresponding to the mutated exon 3 and a reverse primer of exon 7. The presence of base 'C' at nucleotide 873, specific for the SMN gene (arrow) demonstrates that the frameshift mutation belongs to SMN.

To determine whether the microdeletion belonged to the SMN gene or to the centromeric copy we performed RT–PCR amplification of RNA from the mother's leukocytes and synthesized specifically the cDNA harboring the microdeletion by using a specific primer for the mutated sequence in exon 3 (primer CD) and a reverse primer in exon 7. Sequencing of this cDNA product showed the sequence characteristic of exon 7 from the SMN gene, indicating that the mutation belongs to this gene (Fig. 2). The same study could not be performed for the affected daughter since no RNA was available due to her early death, which did not allow us either to take a muscle biopsy nor to establish a lymphoblastoid cell line. Therefore we used long range PCR to amplify the genomic DNA of the patient and parents from exon 3–8 with either the specific primer (CD) for the mutated exon 3 or a primer for the normal sequence (CND) and a reverse primer in exon 8. PCR products of 10.5 kb were obtained (Fig. 3) and then used for SSCP analysis following a second PCR amplification with nested primers of exons 7 and 8 (Fig. 4). The SSCP pattern of exon 7 and 8 of the mother and affected daughter, obtained by amplification of the mutated gene, corresponded to that of the SMN gene, confirming that the microdeletion belongs to this gene. On the other hand, SSCP analysis of long PCR products obtained with the primer for the normal exon 3 showed only the presence of the centromeric gene(s) in the patient, providing evidence that the patient has no intact SMN gene. These data suggest that the infant had a paternal chromosome deleted for the SMN gene.

Interestingly, SSCP analysis of the mother's exons 7 and 8, showed strong bands corresponding to the SMN gene and very faint bands, barely visible, corresponding to the centromeric copy (Fig. 4). The same pattern was consistently observed by restriction digest assay with *Dra*I and sequence analysis of exon 7 (data not shown).

Genotype analysis

In the attempt to gain further information on the molecular organization at the SMA locus in the proposita we investigated the presence of the NAIP gene and performed segregation analysis of polymorphic markers linked to 5q13. Study of the patient's NAIP gene showed the presence of exon 5 which is specific for the functional NAIP gene and absent in a variable

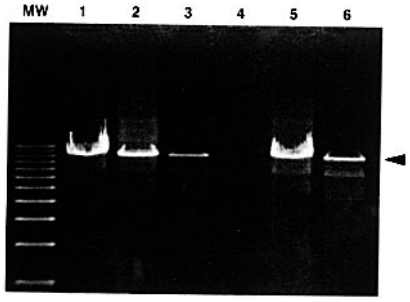

Figure 3. Long range PCR amplification from exon 3 to exon 8. PCR amplification was performed using primer CND (corresponding to the non-deleted exon 3) (lanes 1–3) or primer CD (corresponding to the deleted exon 3) and an exon 8 reverse primer (lanes 4–6). Control individual (lanes 1 and 4), mother (lanes 2 and 5), proband (lanes 3 and 6). Arrowhead indicates the 10.5 kb amplification products. Specific DNA amplification reaction from the deleted exon 3 to exon 8 is strongly supported by the absence of product in the control. MW, molecular weight marker.

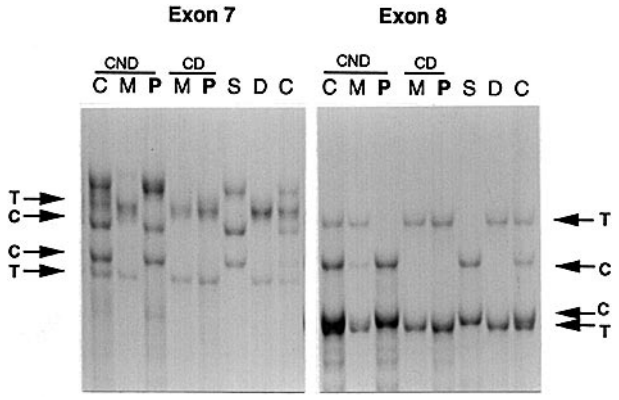

Figure 4. SSCP analysis of nested PCR amplification of exons 7 and 8, using as DNA template long range PCR products obtained either with the primer (CND), corresponding to the non-deleted exon 3 (lanes marked CND), or the primer specific for the deleted exon 3 (lanes marked CD), together with reverse primers. P, proband; M, mother; C, control individual; S, SMA patient deleted for SMN exons 7 and 8; D, control individual lacking the centromeric copy. Arrows indicate the bands corresponding to the SMN gene (T) or the centromeric copy (C).

number of pseudogenes mapping in the SMA critical region. Thus, detection of exon 5 is usually taken as evidence for the existence of at least one active copy of this gene (15).

Analysis of a recently identified variant in SMN exon 2a, which is specific for the centromeric copy (18) showed the presence of this variant only in the proposita's father. Neither the proposita nor her normal brother received this centromeric gene carrying the base difference.

Segregation analysis was performed using polymorphic markers from the SMA region including the multicopy markers C212 and Ag1-Ca (C272) which are both located 5′ to SMN and its centromeric homologue (19). Haplotype construction is not straightforward due to the occurrence of more than one allele per chromosome at the multicopy loci. The putative haplotypes and segregation of alleles at the most informative polymorphic loci are depicted in Figures 5 and 6. The patient and her brother received the same paternal allele at the close distal locus *D5S557*

Figure 5. Pedigree and haplotypes of family members. The figure depicts the likely arrangement at the SMA locus of the copies of the centromeric homologue and SMN gene. Cen-copy, centromeric copy; fm, SMN gene with the frameshift mutation in exon 3.

demonstrating that the father has transmitted the same chromosome 5 fragment to both his offspring, as already suggested by the absence of the paternal exon 2a variant in the sibs. This is further supported by the absence of the paternal allele 2 of C212 in both sibs. However, the son has received the paternal allele 2 of locus Ag1-CA (C272) which has not been inherited by the affected daughter (non paternity was excluded), suggesting a *de novo* deletion in the patient's DNA of at least one locus Ag1-CA. The proposita has two Ag1-CA alleles, allele 1 from her mother and allele 3 likely from her father. Similarly, she has two C212 alleles, one from her mother and the other presumably from the father. Taken together, the pattern of inheritance at the multicopy loci C212 and Ag1-CA suggests that the daughter has one maternal contribution, associated with the SMN gene carrying the frameshift mutation, and the other (paternal) contribution consisting of one copy-gene (Fig. 5).

SSCP variant of exon 3

Sequencing of the patient's SMN exon 3, amplified with primers (E3F/E3R) flanking the site of the frameshift mutation, showed only the sequence with the microdeletion (Fig. 1). However, by using a more distal reverse primer (E3R2) both the normal and the mutated sequence was amplified suggesting that base difference in the non mutated gene prevented annealing of the primer E3R. Indeed, a synonymous CAG→CAA base substitution was detected in codon 154. Sequence analysis of the other family members showed that the father was heterozygous for this base difference while his son had only the variant form (CAA). Thus, in our family the published exon 3 sequence was present only in the SMN gene harboring the microdeletion in the proposita and mother and in the chromosome of the father which was not transmitted to his offspring. The base change can be easily detected by SSCP analysis (Fig. 7A) and/or digestion of PCR amplification products with the restriction enzyme *Bsm*I which

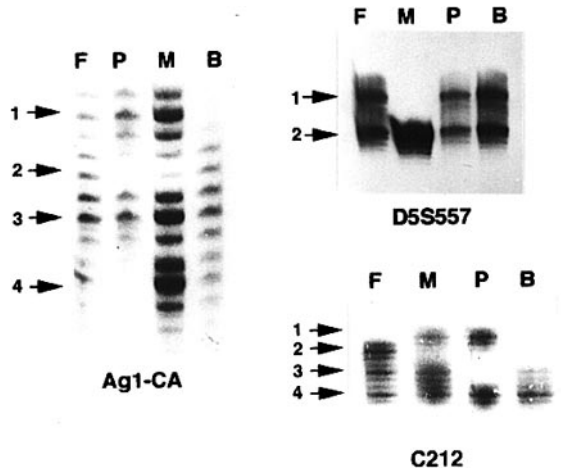

Figure 6. Segregation of alleles at the multicopy loci Ag1-CA and C212. *D5S557* maps distal to the SMA locus. Microsatellite analysis of the loci Ag1-CA and C212 were repeated several times and stronger bands were found consistently for allele 3 of Ag1-CA and allele 4 of C212 in the brother (B) suggesting two doses of these alleles. Similarly, the father (F) presumably has two doses of allele 3 of Ag1-CA. The patient (P) consistently has two bands of equal intensity. Mother (M). Arrows indicate the alleles arbitrarily assigned with consecutive numbers.

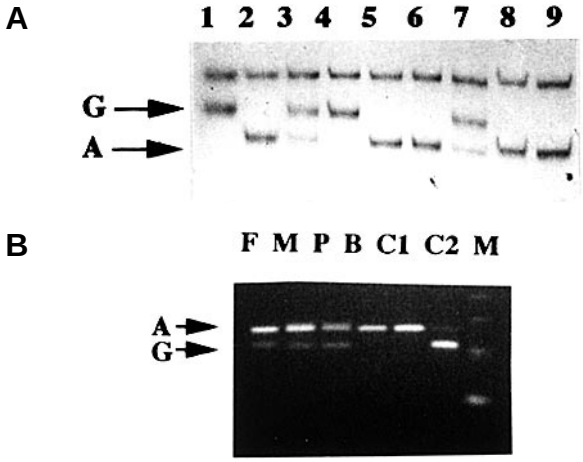

Figure 7. (**A**) Analysis of polymorphism in exon 3 by SSCP analysis. Lanes 1 and 4, homozygotes G/G; lanes 2, 5, 6, 8 and 9, homozygotes A/A; lanes 3 and 7, heterozygotes G/A. (**B**) Restriction digest assay in the family members and controls. Father (F), mother (M) and proband (P) are heterozygous while the brother (B) is homozygous A/A. C1 and C2, control individuals homozygous A/A and G/G, respectively. Arrows indicate sequences containing base G and A, respectively.

cuts the variant form but not the CAG containing sequence (Fig. 7B). Study of 24 SMA type I–III patients deleted for SMN, 31 carriers and 20 controls, demonstrated homozygosity for the variant codon CAA in 58, 23 and 10%, respectively and homozygosity for codon CAG in 4, 13 and 30%, respectively. These data imply that codon 154 is frequently polymorphic, both in the SMN and the centromeric copy-gene (Fig. 3), and that the CAA codon is more frequently found in the affected individuals.

DISCUSSION

We have found exon 7 of the survival motor neuron gene (SMN) homozygously deleted in >96% of SMA type I–IV patients. The five patients who were not deleted for exon 7 were studied for the presence of mutations in the other seven exons by SSCP analysis. No band shifts were found in four of them. Since SSCP analysis does not allow us to detect 100% of point mutations, sequencing of the entire coding sequence in these clinically confirmed patients is being performed.

In one of the undeleted SMA type I patients we detected a 5 bp microdeletion (GAAGT) in exon 3 of the SMN gene at codons 147–148. The mutation introduces a premature stop codon (TAA) four nucleotides downstream resulting in a truncated protein. From the SSCP pattern of long range PCR amplification products it could be deduced that the mutation occurred in the SMN gene and that the patient does not have an intact copy of the SMN gene.

During the study of exon 3 in our family we have identified a silent mutation at codon 154. This is the first frequent polymorphism identified in the coding sequence of both the SMN and the centromeric copy. Segregation analysis of this polymorphic codon, which can be easily performed by SSCP and/or restriction digest assay, could be useful for studying the parental contribution and the extent of deletion in SMA families.

The present mutation is the second frameshift mutation in SMN reported so far in SMA patients. The previous reported microdeletion (AGAG) also occurred in exon 3 at codons 133–134 (16). The localization of both frameshift mutations in exon 3 could suggest that this domain contains a hot spot of hypermutability. The earlier described deletion was detected in four patients from unrelated Spanish families, one had SMA type I, one type II and two type III. The deletion described here was identified in a severely affected newborn who died 42 days after birth. In our cohort of type I patients only two other infants died at the age of 6 weeks while the mean age of death is 6 months.

It has been suggested that the NAIP gene, when deficient or absent, contributes to the SMA phenotype (16,20–21). Our patient did not show a homozygous deletion of NAIP exon 5 suggesting the presence of at least one full-length gene. The finding of one, presumably functional, NAIP gene in the proposita is not unexpected since no gross deletion had occurred at the close SMN locus in the maternal chromosome.

An alternative hypothesis to explain the different phenotypes is that the clinical expression may be influenced by the presence of a variable number of SMN or centromeric copies, supported by the correlation between the severity of phenotype and the number of observed alleles at the multicopy loci (3,5). Based on the data obtained by densitometric analysis of SSCP bands of exon 7, Velasco *et al.* (8) have suggested the presence of a higher number of copy-genes in patients of type II and III than in those of type I.

Haplotype analysis in our family showed a *de novo* deletion of at least one paternal allele Ag1-CA. Ag1-CA corresponds to C272 and is located at only 463 bp upstream to the ATG translation start site of SMN and its homologous copy while C212 maps ~11 kb proximal to Ag1-CA (19). Deletion of an Ag1-CA allele and/or a C212 allele generally indicates that at least part of the SMN gene is also deleted. The precise segregation of alleles at the multicopy microsatellite marker loci Ag1-CA and C212 is difficult to establish. In our family, the patient has two Ag1-CA and two C212 alleles. One of each must be of maternal origin, located upstream to the SMN gene harboring the frameshift

mutation. The others are presumably of paternal origin considering the pattern of inheritance in all family members, although we cannot rule out the possibility that these alleles derive from the mother. In any case, given that the proposita has no other SMN gene, these Ag1-CA and C212 alleles should be located 5′ to one single centromeric gene.

Study of the mother's exons 7 and 8 consistently showed a very low intensity of bands corresponding to the centromeric gene (Fig. 4), possibly due to a PCR artefact. Alternatively, we may speculate on the possibility of mosaicism caused by a somatic mutation. Since the SMN gene is located in an unstable region containing repetitive elements, pseudogenes and retrotransposon sequences (22) and since at least 1% of patients are due to *de novo* mutations, it might be hypothesized that deletions and rearrangements could also occur in somatic cells, including motor neurons during development, and that this phenomenon may be one of the mechanisms influencing, at least in part, the clinical variability.

Taken together, genotype and mutation analysis in this family suggest that the proposita has inherited one SMN gene resulting in a truncated protein from the mother and one centromeric homologue from the father (Fig. 5). The centromeric gene, but not SMN, undergoes alternative splicing producing transcripts lacking exon 7 which likely leads to a reduced concentration of the full-length transcripts (3). Thus, we suggest that the severe phenotype in our patient is the consequence of the reduced number of centromeric genes in addition to the frameshift mutation.

MATERIALS AND METHODS

Deletion analysis of the SMN gene

PCR amplification of DNA from peripheral leukocytes and SSCP analysis were performed as previously described (23). Exons 7 and 8 were amplified using the primer pairs reported by Lefebvre *et al.* (3).

Deletion analysis of NAIP exon 5

Multiplex PCR of exons 5 and 13 was carried out using the primer pairs 1863+1864 and 1258+1343, respectively (15). Exon 5 is present only in the functional gene while exon 13 is present also in pseudogene copies. Both exons were coamplified to rule out PCR failure. Amplified DNA was electrophoresed onto a 1.2% agarose gel and stained with ethidium bromide.

SMN exon 3 analysis

SSCP of exon 3 of SMN and copy-gene was performed following PCR amplification using the primers E3F (5′-CGAGATGATAGTTTGCCCTCTT-3′) and E3R (5′-CTTACCTCTTGAGCATTCTGTT-3′) and the annealing temperature of 62°C. Polymorphism in codon 154 of the SMN and copy-gene was analyzed by SSCP and direct sequencing as described above but using as reverse primer E3R2 (5′-CTCATCTAGTCTCTGCTTCC-3′) and the annealing temperature of 62°C. For specific PCR amplification of exon 3 harboring the microdeletion we used the forward primer CD (5′-TCCCCAATCTGTAGCTAATAATAT-3′), while the non-deleted exon 3 was amplified with primer CND (5′-TCCCCAATCTGTGAAGTAGCTAAT-3′); as reverse primer either E3R or E3R2 was chosen. Annealing temperature was 63°C. Nucleotide sequence of normal and mutated PCR amplified exon 3 was determined using the Sequenase PCR product sequencing kit (Amersham).

Analysis of polymorphism in exon 3

The polymorphic site at codon 154 was revealed by SSCP analysis of PCR amplified exon 3 with primers E3F and E3R2. Alternatively, the base difference can be detected by digestion of PCR products of exon 3 with the restriction enzyme *Bsm*I (Boehringer) which cuts the sequence containing the CAG codon but not that containing CAA.

Long range PCR and SSCP of nested PCR products

Amplification from exon 3 to 8 was performed using either the primer CND (corresponding to the non-deleted exon 3) or primer CD (specific for exon 3 containing the microdeletion) together with the reverse primer C1120 of exon 8. PCR amplification products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. The long range PCR products were used for nested PCR amplification of exon 7 with primers R111/C770 and of exon 8 with primers C960/C1120. These nested PCR products were subjected to SSCP analysis.

RT–PCR

Total RNA from peripheral blood lymphocytes of the proband's mother was isolated using the procedure described elsewhere (24). First-strand cDNA synthesis from total RNA, primed with oligo-dT 20 μg/μl (Pharmacia) was carried out with MMLV-reverse transcriptase (Gibco BRL) in a volume of 20 μl. The single-stranded cDNAs were PCR amplified using 50 pmol forward (CD) and reverse primer C770 (5′-TAAGGAATGTGAGCACCTTCCTTC-3′), annealing temperature 63°C. The PCR amplified cDNA was sequenced using primer C618 (5′-CTCCCATATGTCCAGATTCTCTTG-3′) and the AmpliCycle kit (Perkin Elmer).

Segregation analysis

For genotyping of family members we used the following microsatellite markers: EF$(TG/AG)_n$ (*D5S125*); cVS19 (*D5S435*); C212 (*D5F149S1-2*); Ag1-CA; *D5S557*; JK53. PCR amplification was performed as described earlier (25), except that primers were labelled with [^{32}P]dCTP.

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