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Intragenic telSMN Mutations: Frequency, Distribution, Evidence of a Founder Effect, and Modification of the Spinal Muscular Atrophy Phenotype by cenSMN Copy Number

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Summary

The autosomal recessive neuromuscular disorder proximal spinal muscular atrophy (SMA) is caused by the loss or mutation of the survival motor neuron (SMN) gene, which exists in two nearly identical copies, telomeric SMN (telSMN) and centromeric SMN (cenSMN). Exon 7 of the telSMN gene is homozygously absent in ~95% of SMA patients, whereas loss of cenSMN does not cause SMA. We searched for other telSMN mutations among 23 SMA compound heterozygotes, using heteroduplex analysis. We identified telSMN mutations in 11 of these unrelated SMA-like individuals who carry a single copy of telSMN: these include two frameshift mutations (800ins11 and 542delGT) and three missense mutations (A2G, S262I, and T274I). The telSMN mutations identified to date cluster at the 3′ end, in a region containing sites for SMN oligomerization and binding of Sm proteins. Interestingly, the novel A2G missense mutation occurs outside this conserved carboxy-terminal domain, closely upstream of an SIP1 (SMN-interacting protein 1) binding site. In three patients, the A2G mutation was found to be on the same allele as a rare polymorphism in the 5′ UTR, providing evidence for a founder chromosome; Ag1-CA marker data also support evidence of an ancestral origin for the 800ins11 and 542delGT mutations. We note that telSMN missense mutations are associated with milder disease in our patients and that the severe type I SMA phenotype caused by frameshift mutations can be ameliorated by an increase in cenSMN gene copy number.

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Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by destruction of α-motor neurons in the anterior horn of the spinal cord. SMA has an estimated incidence of 1/10,000 live births, with a carrier frequency of ~1/50 individuals (Pearn 1980; Melki et al. 1994). Childhood-onset SMA is classified into three groups, on the basis of age at onset and clinical course (Munsat and Davies 1992). Type I SMA (Werdnig-Hoffmann disease; MIM 253300) is the most severe form, with onset at <6 mo of age and death usually occurring by 2 years of age. Type II SMA (MIM 253550) is of intermediate severity: onset occurs at <18 mo of age, and patients never gain the ability to walk. Type III SMA (Kugelberg-Welander disease; MIM 253400) is the mildest form of the disease: onset occurs at >18 mo of age, and patients are able to both stand and walk on their own.

All three forms of childhood SMA have been mapped, by linkage analysis, to chromosome 5q11.2-q13.3 (Brzustowicz et al. 1990; Melki et al. 1990; Daniels et al. 1992; Simard et al. 1992; MacKenzie et al. 1993; Brahe et al. 1994; Burghes et al. 1994*a;* Wirth et al. 1994, 1995), a region of complex genomic organization that contains numerous repeated sequences, including polymorphic markers and genes (Francis et al. 1993; Kleyn et al. 1993; Carpten et al. 1994; Melki et al. 1994; Lefebvre et al. 1995; Roy et al. 1995; Thompson et al. 1995). Three genes have been identified and characterized in the SMA critical region: the survival motor neuron (SMN) gene (Lefebvre et al. 1995), the gene for neuronal apoptosis inhibitory protein (Roy et al. 1995; Thompson et al. 1995), and p44, a subunit of the gene for the basal transcription factor TFIIH (Bürglen et al. 1997; Carter et al. 1997).

The SMN gene is present in two nearly identical copies on 5q, telomeric SMN (telSMN) and centromeric SMN (cenSMN), which can be distinguished by single-base changes in exons 7 and 8 (Lefebvre et al. 1995; van der Steege et al. 1995). Exon 7 of the telSMN gene is not

detectable in ~95% of SMA patients, owing to either deletion of telSMN or conversion of telSMN sequences to cenSMN (Hahnen et al. 1995; Lefebvre et al. 1995; Rodrigues et al. 1995; Hahnen et al. 1996; van der Steege et al. 1996; Velasco et al. 1996; DiDonato et al. 1997). Loss of the cenSMN gene does not cause SMA; however, increased cenSMN gene copy number, which can occur as a result of gene conversion events from telSMN to cenSMN, has been shown to be associated with a milder SMA phenotype (Burghes 1997; Campbell et al. 1997; DiDonato et al. 1997; McAndrew et al. 1997). The fact that exon 7 of the telSMN gene is homozygously absent in a large majority of SMA patients has enabled the development of an effective PCR-based assay for the molecular diagnosis of SMA (Lefebvre et al. 1995; van der Steege et al. 1995). In addition, diagnosis of SMA carriers is now possible, by use of a quantitative PCR-based assay for determination of telSMN copy number (McAndrew et al. 1997). In SMA patients who retain the telSMN gene, a limited number of other intragenic telSMN mutations have been identified (Bussaglia et al. 1995; Lefebvre et al. 1995; Brahe et al. 1996; Bürglen et al. 1996*b;* Parsons et al. 1996, 1998; Hahnen et al. 1997; McAndrew et al. 1997; Rochette et al. 1997; Talbot et al. 1997*a*), providing strong evidence for confirmation of the role of the SMN gene as the primary SMA-determining gene.

Both the cenSMN and telSMN genes have been transcribed, and alternative splicing of these transcripts has been demonstrated. The telSMN gene produces primarily full-length SMN transcripts, whereas the cenSMN gene produces more alternatively spliced isoforms (lacking exons 5 and/or 7) and less full-length SMN transcripts (Gennarelli et al. 1995; Lefebvre et al. 1995). Only expression of full-length SMN transcripts can be detected at the protein level. The reduction of SMN-protein levels in SMA patients has been found to be directly correlated with the severity of the SMA phenotype (Coovert et al. 1997; Lefebvre et al. 1997). The detection of SMN protein in SMA patients who lack the telSMN gene indicates that the cenSMN gene does produce protein, although in significantly lower amounts than the telSMN gene.

Although several intragenic telSMN mutations have been reported in SMA patients, a more comprehensive survey of these mutations is essential for providing insight into important structural and functional domains of the SMN protein. Recent experiments have suggested a role for the SMN protein in spliceosomal small nuclear ribonucleoprotein (snRNP) biogenesis (Liu and Dreyfuss 1996; Fischer et al. 1997; Liu et al. 1997; Mattaj 1998), but information regarding critical regions of the SMN protein remains limited. Since ~95% of SMA patients are homozygously lacking the telSMN gene, the relatively rare intragenic telSMN mutations are valuable for defining these critical domains. In addition, these mutations may help to further define the correlation between genotype and phenotype in SMA and to elucidate the role of the cenSMN gene in modifying the severity of the disease. A more extensive telSMN mutation survey also could have direct clinical utility; if common intragenic mutations are found in this population of SMA patients who retain a copy of the telSMN gene, assays for detection of these mutations can be designed to increase the sensitivity of SMA molecular diagnosis.

The purpose of our study was to determine the frequency, type, and distribution of intragenic telSMN mutations and their relation to SMA phenotype. In this article, we describe the results of screening for small mutations in the entire coding region of the telSMN gene, in a large population of patients referred for SMA diagnostic testing. Our mutation-screening protocol involved an initial quantitative PCR assay of telSMN copy number, for identification of likely SMA compound heterozygotes (individuals lacking one copy of telSMN and having another mutation in their other copy), followed by heteroduplex analysis for telSMN mutation detection.

Patients and Methods

Patient Selection and DNA Isolation

A total of 414 patients, referred to the Ohio State University (OSU) Molecular Pathology Laboratory for diagnostic SMA testing, were analyzed, to identify potential compound heterozygotes. These patients did not necessarily conform to all clinical criteria defined by the SMA consortium (Munsat and Davies 1992). A small number of patient samples were collected for prior genetic studies; these patients did meet the established diagnostic criteria for SMA. DNA from all patients was isolated from peripheral venous blood, by use of a salting-out protocol (Miller et al. 1988).

telSMN Exon 7 Deletion Analysis

The presence or absence of telSMN exon 7 was determined by use of a PCR-based protocol, described elsewhere (Lefebvre et al. 1995; van der Steege et al. 1995), in which *Dra*I restriction-enyzme digestion of SMN exon 7 PCR product allows the cenSMN and telSMN genes to be distinguished.

telSMN and cenSMN Gene Dosage Analysis

In patients who retained at least one copy of telSMN, the copy number of the telSMN and cenSMN genes was determined by use of a quantitative PCR assay, as described elsewhere (McAndrew et al. 1997). This assay utilizes an exon of the cystic fibrosis transmembrane regulator (CFTR) gene as a standard, to determine the copy

Table 1

PCR Primers Used for Heteroduplex Analysis of telSMN

Exon	Size (bp)	Primer Sequence (5′→3′)	
		Sense	Antisense
1	189	GCCGGAAGTCGTCACTCTT	GGGTGCTGAGAGCGCTAATA
2A	203	CTGATTAAACCTATCTGAACATG	CGTATGTTATCAATTCCTTTCCA
2B	218	CTGTGCACCACCCTGTAACATG	AAGGACTAATGAGACATCC
3	320	CGAGATGATAGTTTGCCCTC	CTCATCTAGTCTCTGCTTCC
4	252	CACCCTTATAACAAAAACCTGC	GAGAGGTTAAATGTCCCGA
5	314	TGAGTCTGTTTGACTTCAGG	TATCAAATTGTATGTGAAAGCA
6	246	CTCCCATATGTCCAGATTCTCTT	AAGAGTAATTTAAGCCTCAGACAG
7	245	AGACTATCAACTTAATTTCTGATC	GTAAGATTCACTTTCATAATGCTG

number of the telSMN and cenSMN genes, and incorporates the use of two internal standards (SMN-IS and CFTR-IS), to monitor the efficiency of the PCR reaction and to ensure that equal amounts of target genomic DNA are added to each tube. Since this test uses CFTR as the standard for determining the telSMN copy number, the significant limitation imposed by use of the cenSMN gene (for which the copy number varies, in both patients and controls) as a reference is avoided.

Heteroduplex Analysis

Patient samples in which telSMN gene dosage analysis revealed only a single copy of telSMN were subjected to heteroduplex analysis. Each SMN coding exon (1, 2A, 2B, and 3–7) was amplified, by PCR, from genomic DNA: the PCR primers used (table 1) were selected on the basis of published intron/exon boundary sequences (Bürglen et al. 1996*a*) and the primer sequences kindly provided by Dr. Judith Melki. The PCR reactions were performed by use of 75 ng each of the appropriate sense and antisense primers, in a 50-μl reaction mixture containing 1 U *Taq* polymerase (Perkin-Elmer), 0.5 mM dNTP, 3 mM $MgCl_2$, and 5 μl 10 × PCR buffer (670 mM Tris, 100 mM β-mercaptoethanol, 166 mM ammonium sulfate, 67 mM EDTA, and 0.5 mg BSA/ml). PCR cycling consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min, with a final extension at 72°C for 8 min. To allow heteroduplex formation, PCR products were heated to 95°C for 5 min and then were incubated at 37°C for 30 min. Fifteen microliters of PCR product was mixed with 2.5 μl 6 × mutation detection–enhancement (MDE) gel loading buffer (FMC) and then was electrophoresed on a 50-cm-high, 0.8-mm-thick MDE gel (FMC), for 15 h at 1,000 V. The gel was stained in a solution of 0.6 × Tris borate–EDTA containing 1 μg ethidium bromide/ml and then was photographed under ultraviolet light.

Subcloning and Sequencing

The PCR and reverse transcription–PCR (RT-PCR) amplification products to be sequenced were subcloned into the TA cloning vector (Invitrogen), in accordance with the manufacturer's instructions. Sequencing of plasmid DNA purified by use of Wizard Minipreps (Promega) was performed with the dsDNA Cycle Sequencing System (Gibco BRL). Sequencing-reaction products were analyzed by use of a 5% denaturing polyacrylamide gel. The gels were transferred onto blotting paper, dried, and exposed to Hyperfilm-MP (Amersham) at −70°C for 12–24 h. Mutations were sequenced on both DNA strands, and multiple subclones were analyzed.

FokI *Restriction-Enzyme Analysis of SMN Exon 1 Subclones*

Exon 1 of the SMN gene was PCR amplified (table 1) from genomic DNA from patients with the A2G missense mutation and from controls and then was subcloned into the TA cloning vector (Invitrogen). After colony PCR of the exon 1 subclones, 18 μl of the resulting 189-bp PCR product was digested with 4 U *Fok*I (New England Biolabs) for 2 h and was electrophoresed on 12% polyacrylamide gels, for 400 V-h. Both the A2G missense mutation and the C→T polymorphism 14 bp upstream of exon 1 create *Fok*I sites; *Fok*I digestion cleaves the 189-bp PCR product 79 bp from the 5′ end in alleles with the A2G mutation and 32 bp from the 5′ end in alleles with the C→T polymorphism.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from peripheral blood lymphocytes or from skeletal muscle, by use of TRIzol Reagent (Gibco BRL). First-strand cDNA synthesis was performed with 2 μg RNA (based on spectrophotometric quantitation) by use of oligo(dT) and Superscript II Rnase H^- Reverse Transcriptase (200 U/μl; Gibco BRL), in accordance with the manufacturer's instructions.

RT-PCR Confirmation of Variant SMN Sequences as Telomeric

In order to determine whether the variant SMN sequences identified in SMA patients were contained within the telSMN or cenSMN genes, RT-PCR analysis was performed. The single-stranded cDNAs synthesized from patient total RNA were PCR amplified, by use of sense and antisense primers selected to encompass both the variant sequence and the exon 7 and exon 8 single-base changes between the telSMN and cenSMN genes, and then were subcloned. These sequence changes allow the centromeric and telomeric copies of exons 7 and 8 to be differentiated by use of *Dra*I (exon 7) or *Dde*I (exon 8) restriction-enzyme digestion (Lefebvre et al. 1995; van der Steege et al. 1995). It then could be demonstrated, by direct sequencing and/or restriction digestion of patient subclones, whether the variant SMN sequences were exclusive to transcripts derived from telSMN or from cenSMN.

For the A2G missense mutation, 75 ng each of sense primer SMNaf (5′-TGC GCA TCC GCG GGT TTG CT-3′) and antisense primer SMNcr (5′-TCA TTT AGT GCT GCT CTA TGC CA-3′) were used to amplify patient cDNA from exons 1–8, by means of reaction-mixture components and PCR conditions identical to those used for the heteroduplex analysis. The resulting RT-PCR products then were subcloned into the TA cloning vector (Invitrogen). Patient subclones containing telSMN exon 7 were identified by PCR and *Dra*I digestion. These telomeric subclones were shown to contain the C→G transversion at position 38, by *Fok*I digestion and electrophoresis on 12% polyacrylamide gels, demonstrating that the A2G mutation occurs in the telSMN gene.

RT-PCR Analysis of the SMN Gene in a Potential SMA Compound Heterozygote

Single-stranded cDNA synthesized from patient and control lymphocyte total RNA was PCR amplified by use of the following overlapping primer sets, selected to span the SMN cDNA: (1) exons 1–3, sense primer SMNaf (5′-TGC GCA TCC GCG GGT TTG CT-3′) and antisense primer SMNar (5′-GAA CAT TTG TCC CCA ACT TTC CA-3′); (2) exons 2B–4, sense primer SMNbf (5′-AAA GCC AAA AGA AGA ATA CTG CAG-3′) and antisense primer 541C460 (5′-GAG AAA GGA GTT CCA TGG AGC AG-3′); (3) exons 4–8, sense primer 541C380 (5′-GTG AGA ACT CCA GGT CTC CTG G-3′) and antisense primer SMNcr (5′-TCA TTT AGT GCT GCT CTA TGC CA-3′); and (4) exons 6–8, sense primer 541C618 (5′-CTC CCA TAT GTC CAG ATT CTC TT-3′) and antisense primer 541C1120 (5′-CTA CAA CAC CCT TCT CAC AG-3′). RT-PCR reaction-mixture components and cycling conditions were identical to those used for the heteroduplex analysis. The resulting RT-PCR products were electrophoresed on 12% polyacrylamide gels, stained with ethidium bromide, and photographed under ultraviolet light.

Semiquantitative RT-PCR Analysis of SMN Transcripts

The transcript of the hypoxanthine phosphoribosyl transferase (HPRT) gene was used as an internal control, to allow analysis and comparison of the SMN-transcript levels of the samples. PCR primers 541C380 and 541C1120 were used for amplification of exons 4–8 of the SMN gene, yielding four possible RT-PCR products (derived from full-length SMN transcripts and isoforms lacking exons 5 and/or 7). Primers for amplification of HPRT (HPRTf, 5′-TGT AAT GAC CAG TCA ACA GG-3′, and HPRTR1, 5′-ATT GAC TGC TTC TTA CTT TTC T-3′) were selected to produce a product of a size similar to (but distinguishably different from) the full-length SMN transcript (Jolly et al. 1983). The two forward primers (541C380 and HPRTf) were end labeled with $\gamma[^{32}P]$-ATP (10 μCi/μl; Amersham). Two microliters of cDNA (equivalent to 0.2 μg total RNA) was amplified, by PCR, in a 25-μl reaction mixture containing 0.5 mM dNTP, 1 U *Taq* polymerase (Perkin-Elmer), 30 ng of each SMN primer, 7.5 ng of each HPRT primer, 2.5 mM $MgCl_2$, and 2.5 μl 10× PCR buffer. Cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 20 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min, with a final extension step at 72°C for 8 min. Ten microliters of the resulting PCR products was combined with 5 μl loading dye (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) and was electrophoresed on a 6% denaturing polyacrylamide gel. The gel was dried and exposed to Hyperfilm-MP (Amersham) for 1–3 d. Densitometry of the bands was performed on a Shimadzu CS-9000, and the ratios between the amounts of the SMN isoforms and the HPRT transcripts were determined for all samples. Each sample was assayed in duplicate.

Ag1-CA Genotyping

PCR primers and reaction conditions used for amplification of the Ag1-CA microsatellite repeat sequence were as described elsewhere (DiDonato et al. 1994).

Results

Over a period of 4 years (1995–98), samples from 414 individuals were sent to our laboratory for molecular diagnosis of SMA. Of these patients, 206 (49.8%) were found to be homozygously lacking telSMN exon 7, by means of the standard PCR-based assay (Lefebvre et al. 1995; van der Steege et al. 1995). The remaining 208 "nondeletion" patients were screened for small

telSMN mutations, by use of a protocol of initial telSMN gene dosage analysis followed by heteroduplex analysis. Dosage analysis of the telSMN gene revealed that 23 (11.1%) of these 208 nondeletion patients possessed only a single copy of the telSMN gene, making them likely compound heterozygotes (patients with one telSMN gene lost to deletion or sequence conversion and one telSMN gene containing another type of mutation). For these 23 patients, the entire telSMN coding region was screened for small mutations, by heteroduplex analysis. Thus far, five intragenic telSMN mutations have been identified in 11 (47.8%) of these 23 patients (fig. 1 and table 2). Four of the mutations have been described previously by our group and by others; two missense mutations, S262I (Hahnen et al. 1997; McAndrew et al. 1997) and T274I (Hahnen et al. 1997), have been found in one patient each, and two frameshift mutations, 800ins11 (Parsons et al. 1996) and 542delGT (Parsons et al. 1998), have been identified in four unrelated patients and in two unrelated patients, respectively (nucleotide positions were numbered as in the study by Lefebvre et al. [1995]).

We now report the most 5′ intragenic telSMN mutation identified to date—a missense mutation (A2G) that results in the replacement of alanine by glycine in the second codon of the telSMN gene. Heteroduplex analysis of SMN exon 1, PCR amplified from genomic DNA from three unrelated patients (two with type III SMA and one with type II SMA), demonstrated abnormal patterns. Sequence analysis of patient exon 1 subclones revealed a C→G transversion at nucleotide 38, producing a substitution of glycine for alanine (A2G) at a residue, in the deduced SMN protein, that is conserved in zebra fish (A.H.M.B., unpublished data). This base change produces a *Fok*I restriction-enzyme site within exon 1 of the SMN gene, allowing other individuals to be screened easily for the C→G transversion, by exon 1 PCR and *Fok*I digestion. The nucleotide change was not observed in 200 normal chromosomes. In order to determine whether the variant exon 1 sequence was contained within the telomeric or centromeric copy of the SMN gene, patient lymphocyte RNA was amplified by RT-PCR, using an exon 1 sense primer and an exon 8 antisense primer, and then was subcloned. Patient subclones containing telSMN exon 7 were identified by *Dra*I restriction-enzyme digestion; *Fok*I restriction-enzyme analysis of exon 1 of these telomeric subclones revealed that the mutant transcripts were derived from telSMN.

Interestingly, all three patients with the A2G missense mutation also had a C→T polymorphism in the telSMN gene, 14 bp upstream of exon 1. This polymorphism, which also creates a *Fok*I cleavage site, was found in only 3 of 100 control individuals tested. In order to determine whether the A2G mutation and the 5′ UTR polymorphism were on the same telSMN allele, exon 1 subclones both from controls and from A2G patients were amplified by colony PCR and were subjected to *Fok*I restriction-enzyme digestion. The *Fok*I digestion

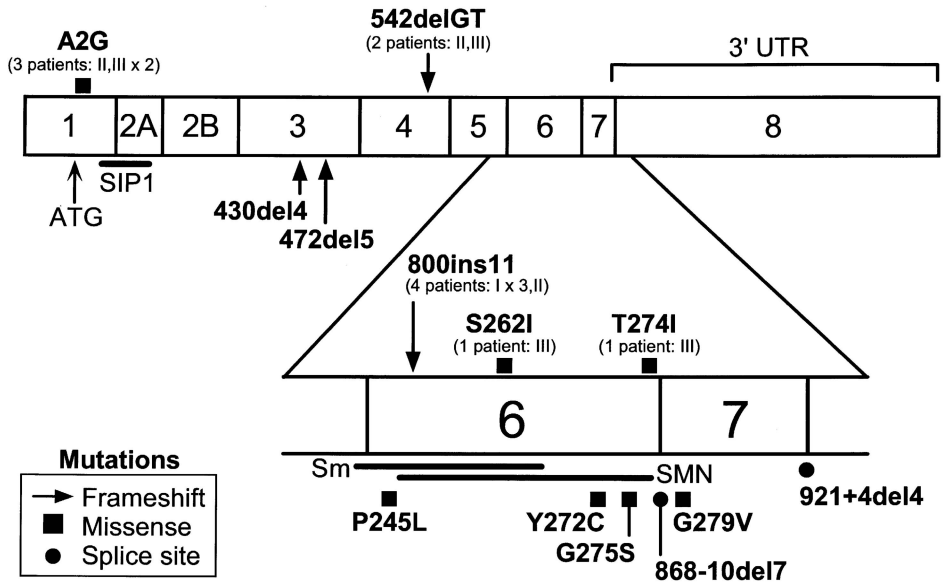

Figure 1 Distribution of reported intragenic telSMN mutations. Mutations identified in the 11 OSU patients (and the resulting SMA phenotypes) are drawn above the telSMN coding region. The S262I and T274I mutations also were reported by Hahnen et al. (1997). SIP1 and Sm protein-binding sites, as well as the SMN oligomerization domain (denoted "SMN"), are indicated by straight lines under the gene. The region of telSMN exons 6 and 7 is shown in greater detail at the bottom of the figure. Refer to table 2 for the authorship of specific mutations.

Table 2

Summary of Small Mutations in the telSMN Gene

Mutation	Location	Nucleotide Change	Effect on Translation	SMA Type	Reference
1	Exon 1	38 C→G	Ala→Gly (A2G)	II, III	This study
2	Exon 3	430 del 4	Frameshift	I, II, III	Bussaglia et al. 1995
3	Exon 3	472 del 5	Frameshift	I	Brahe et al. 1996
4	Exon 4	542 del GT	Frameshift	I, II	Parsons et al. 1998
5	Exon 6	767 C→T	Pro→Leu (P245L)	III	Rochette et al. 1997
6	Exon 6	800 ins 11	Frameshift	I, II	Parsons et al. 1996
7	Exon 6	818 G→T	Ser→Ile (S262I)	III	Hahnen et al. 1997; McAndrew et al. 1997
8	Exon 6	848 A→G	Tyr→Cys (Y272C)	I	Lefebvre et al. 1995; Rochette et al. 1997
9	Exon 6	854 C→T	Thr→Ile (T274I)	II, III	Hahnen et al. 1997; this study
10	Exon 6	856 G→A	Gly→Ser (G275S)	III	Bürglen et al. 1996*b*
11	Intron 6	868-10 del 7	Splicing defect?	…	Lefebvre et al. 1995
12	Exon 7	869 G→T	Gly→Val (G279V)	I	Talbot et al. 1997
13	Intron 7	921+4 del 4	Splicing defect?	…	Lefebvre et al. 1995

products then were electrophoresed on 12% polyacrylamide gels (fig. 2). *Fok*I digestion cleaves the full-length 189-bp exon 1 PCR product 79 bp from the 5′ end in alleles with the A2G mutation and 32 bp from the 5′ end in alleles with the C→T polymorphism. In all three SMA patients with the A2G missense mutation, *Fok*I digestion and PAGE revealed fragments of 32, 47, and 110 bp, confirming that both the mutation and the polymorphism are present on the same telSMN allele and providing evidence for a founder chromosome for this mutation. In order to further investigate the possibility that these intragenic telSMN mutations occur on common genetic backgrounds, Ag1-CA genotyping (DiDonato et al. 1994) was performed on our patients with the A2G, 800ins11, and 542delGT mutations. These family studies were hampered by the fact that limited DNA samples were available from other related individuals; however, each of these three intragenic telSMN mutations appeared to be found on common Ag1-CA alleles. Although incomplete, this Ag1-CA marker data supports an ancestral origin for the 800ins11 and 542delGT frameshift mutations, as well as for the A2G missense mutation.

Although only a relatively small number of intragenic telSMN mutations have been identified, many of them have now been found in multiple unrelated SMA patients. All five telSMN mutations identified in our SMA patient population have now been found in more than one individual (fig. 1). Three of the mutations have been identified more than once in our OSU patient population: the A2G missense mutation has been found in three patients, 800ins11 in four patients, and 542delGT in two patients. Although seen only once in our patients, the two other missense mutations, S262I and T274I, have been identified in another laboratory (Hahnen et al. 1997). The intragenic mutations identified thus far cluster at the 3′ end of the telSMN gene; of the five mutations identified in our patient population, three occur within exon 6 (fig. 1). In fact, 9 of the 13 small telSMN mutations identified by us and by others occur in exons 6 and 7 of the gene; other than the A2G missense mutation described in this article, only frameshift mutations have been identified upstream of exon 6 (table 2).

As a result of not finding intragenic telSMN mutations in 12 of 23 SMA-like individuals with a single copy of

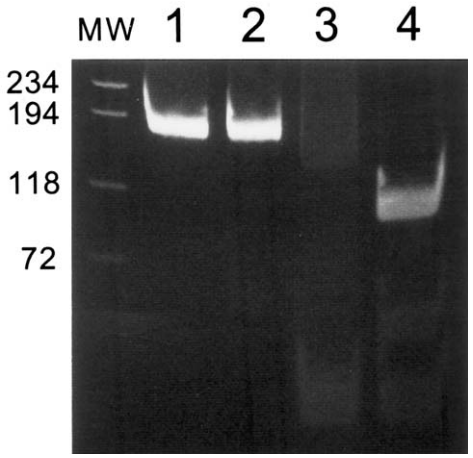

Figure 2 *Fok*I restriction-enzyme analysis of SMN exon 1 subclones. Lane 1, Uncut 189-bp exon 1 colony-PCR product. Lanes 2–4, Colony-PCR products digested with *Fok*I. The control subclone in lane 2 does not contain either the mutation or the polymorphism and, consequently, was not cut by *Fok*I. The control subclone in lane 3 has the polymorphism and was cleaved into 32-bp and 157-bp fragments. An A2G-patient subclone is present in lane 4; as with the other two A2G patients, the polymorphism is present on the same SMN allele as the point mutation, resulting in fragments of 32, 47, and 110 bp after *Fok*I digestion. Lane MW, ΦX174/*Hae*III molecular-weight marker. Bands representing sizes of 234, 194, 118, and 72 bp are labeled.

telSMN, we decided to pursue further studies at the RNA level. Some types of telSMN mutations, such as intronic variations affecting telSMN splicing and whole-exon duplications or deletions in telSMN, would be missed by our screening protocol using heteroduplex analysis of the SMN coding region; one method of screening for these other types of mutations is by RT-PCR analysis of SMN transcripts. In 1 of our 12 potential SMA compound heterozygotes without an identified telSMN mutation (patient 7620), lymphocyte RNA was amplified by RT-PCR using primers spanning the SMN transcript and was electrophoresed on 12% polyacrylamide gels. The resulting RT-PCR band patterns were identical to those found in normal controls; no abnormally sized SMN transcripts were identified, and no SMN isoforms were found to be absent. Unfortunately, RNA is currently unavailable from the other potential compound heterozygotes; thus, these individuals have not yet been screened in this manner.

In addition, mutations in the telSMN promoter region or in other sequences affecting transcription of the telSMN gene could account for ~50% of the potential SMA compound heterozygotes in whom no telSMN mutation was found by heteroduplex analysis. In order to investigate this possibility, semiquantitative RT-PCR was performed on patient 7620, as well as on SMA patients and normal controls (fig. 3). This RT-PCR assay used the transcript of the HPRT gene as an internal control, to allow analysis and comparison of the SMN-transcript levels of the samples. PCR primers located within exons 4–8 of the SMN gene were used to amplify four possible RT-PCR products (derived from full-length SMN transcripts and from isoforms lacking exon 7, exon 5, or both exons 5 and 7); primers selected to amplify the HPRT transcript were included in the same PCR reaction. Densitometry of the resulting bands was performed, and the ratios between the amounts of the SMN isoforms and the HPRT transcripts were determined. By use of this assay, potential SMA compound heterozygote 7620 (fig. 3, lane 5) was not found to have a significantly reduced amount of full-length telSMN transcript (SMN_{full}/HPRT transcript ratio of 0.89), providing no support for the theory that this patient might have a mutation-altering transcription of the telSMN gene. Polymorphisms in the SMN promoter region have been shown to alter expression of the SMN gene (U. R. Monani, personal communication), which might explain the large difference in levels of full-length SMN transcripts, between our two normal controls (fig. 3, lanes 1 and 6), who have SMN_{full}/HPRT ratios of 0.74 and 2.45. This assay also appears to indicate that the two missense mutations A2G and S262I work by different pathogenic mechanisms. The patient with the A2G mutation has a large amount of full-length SMN transcript (SMN_{full}/HPRT ratio of 1.76), indicating that the mutation's deleterious effect might be occurring at the protein level. In contrast, the patient with the S262I mutation has a reduced amount of full-length SMN mRNA (SMN_{full}/HPRT ratio of 0.28), raising the possibility that this mutation causes its damage at the level of transcription. The S262I mutation has been demonstrated to result in reduced SMN self-association (Lorson et al. 1998).

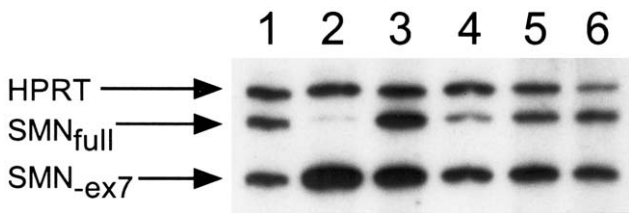

Figure 3 Semiquantitative RT-PCR analysis of SMN transcripts. The HPRT RT-PCR product is present at the top of the gel, with products amplified from full-length SMN transcripts (SMN_{full}) and the SMN isoform lacking exon 7 (SMN_{-ex7}) below. Lanes 1 and 6, Normal controls (SMN_{full}/HPRT ratios of 0.74 and 2.45, respectively). Lane 2, Patient with type II SMA who homozygously lacks telSMN. Lanes 3 and 4, SMA patients with telSMN missense mutations A2G (SMN_{full}/HPRT ratio of 1.76) and S262I (SMN_{full}/HPRT ratio of 0.28), respectively. Lane 5, Potential SMA compound heterozygote in whom no telSMN mutation has been identified (SMN_{full}/HPRT ratio of 0.89).

Attempts to correlate intragenic telSMN mutations with SMA phenotypes are complicated by the effect of the other telSMN allele, which may be either deleted or converted. Despite this fact, several preliminary conclusions regarding SMA genotype-phenotype correlation can be drawn from our patients with small telSMN mutations. In our patients, telSMN missense mutations appear to be associated with relatively mild SMA phenotypes; the A2G mutation was found in two patients with type III SMA and in one patient with type II SMA, and the S262I and T274I mutations were each identified in a patient with type III SMA. In contrast, frameshift mutations in telSMN were found in much more severely affected patients; the 800ins11 mutation was found in three patients with type I SMA and in one patient with type II SMA, and the 542delGT mutation was present in one patient with type I SMA and in one patient with type II SMA. In SMA patients who homozygously lack the telSMN gene, the cenSMN gene copy number has been demonstrated to be correlated with SMA phenotype: an increased number of copies of cenSMN—which can occur as a result of gene conversion events, from telSMN to cenSMN—is more often associated with a mild SMA phenotype (Burghes 1997; Campbell et al. 1997; DiDonato et al. 1997; McAndrew et al. 1997). Analysis of cenSMN copy number in our patients with frameshift mutations provides support for this theory: with regard to mutations 800ins11 and 542delGT, patients with an increased cenSMN copy number had sig-

nificantly less severe SMA phenotypes (table 3). Of the four patients with the 800ins11 mutation, three had two copies of cenSMN and were very severely affected (type I SMA); the fourth individual had three copies of cenSMN and was more mildly affected (type II SMA). Similarly, with regard to the 542delGT mutation, the patient with type I SMA had two copies of cenSMN, and the patient with type II SMA had three copies of cenSMN. The cenSMN–copy number data from our three patients with the A2G missense mutation were not informative; all three individuals possess a single copy of the cenSMN gene.

Table 3

Relationship between cenSMN Copy Number and SMA Phenotype in Patients with Frameshift telSMN Mutations

TelSMN Mutation and Patient Number	No. of CenSMN Copies	SMA Type
800ins11:		
4756	2	I
4961	2	I
5749	2	I
7023	3	II
542delGT:		
5954	2	I
7056	3	II

Discussion

Frequency of Intragenic telSMN Mutations

Of the 414 patients referred to our laboratory for diagnostic SMA testing, 206 (49.8%) were found to be homozygously lacking telSMN exon 7 and were given a positive molecular diagnosis of SMA. For the remaining 208 patients (50.2%), a quantitative PCR assay for determination of telSMN gene copy number (McAndrew et al. 1997) was performed. In addition to allowing the molecular diagnosis of SMA carriers, this test also enables us to efficiently screen patients with SMA-like clinical symptoms, in order to help distinguish SMA compound heterozygotes from non-5q cases (Rochette et al. 1997). Since telSMN is homozygously absent in ~95% of 5q SMA cases, almost all of the remaining 5q SMA individuals should be compound heterozygotes (lacking one copy of telSMN and having a different mutation in their remaining copy of the gene), according to Hardy-Weinberg equilibrium. Once an SMA-like patient with one copy of the telSMN gene has been identified, the probability of a 5q SMA diagnosis is increased significantly. However, it is still possible that the individual may be an SMA carrier whose symptoms are caused by another neuromuscular disorder. We therefore used heteroduplex analysis for mutation screening of the remaining copy of telSMN. By use of this initial quantitative screen for telSMN gene copy number, the number of patients who require mutation analysis can be reduced drastically; we conducted heteroduplex analysis only on the 23 patients (11.1%) who were found to have a single copy of telSMN, instead of on all 208 deletion-negative individuals. For the remaining 185 (88.9%) of these 208 deletion-negative SMA-like patients, the telSMN gene dosage is normal, and 5q SMA is effectively ruled out as a diagnosis; for these cases, the physician should consider other neuromuscular diseases. This telSMN gene dosage–based screening strategy would not be as useful in the diagnosis of SMA in consanguineous families or in groups exhibiting a significant founder effect, since individuals who are homozygous for a nondeletion telSMN mutation would be more common among those groups (Bussaglia et al. 1995). If we consider the 23 patients analyzed as having a positive molecular diagnosis of 5q SMA, then 206 (90%) of 229 SMA-positive patients in our population were homozygously lacking telSMN exon 7, whereas 23 (10%) of 229 patients were compound heterozygotes. Various studies have determined the proportion of SMA patients who homozygously lack telSMN (as a result of either deletion or sequence conversion) to be ~95% (Cobben et al. 1995; Hahnen et al. 1995, 1996; Lefebvre et al. 1995; Rodrigues et al. 1995; van der Steege et al. 1996; Velasco et al. 1996; DiDonato et al. 1997); our numbers are in approximate agreement with this data, especially in view of the possibility that some of these 23 individuals with one copy of telSMN are SMA carriers with another neuromuscular disease.

By use of heteroduplex analysis, telSMN mutations were identified in 11 (47.8%) of 23 possible compound heterozygotes. There are many possible reasons to explain why telSMN mutations were not identified in the other 12 patients: (1) some of these individuals may be carriers of a telSMN-deleted or -converted allele and may not be actual SMA patients; (2) the promoter and 3′ UTR regions were not analyzed extensively; (3) intronic sequences may play a significant role in disease expression; (4) whole-exon duplications or deletions would not be detected by heteroduplex analysis; and (5) heteroduplex analysis does not have 100% sensitivity in screening for sequence variations. Although telSMN mutations were found only in approximately half of the patients with a single copy of telSMN, the detection rate varied dramatically by SMA phenotype: mutations were identified in all four individuals with type I SMA (three of whom had the 800ins11 mutation) but in only 7 (36.8%) of the 19 patients with milder SMA phenotypes. Since whole-exon mutations would not be predicted to disrupt the translational reading frame, they could produce a mild SMA phenotype; similarly, intronic mutations affecting telSMN transcript splicing also could allow some telSMN protein function. Perhaps the lower

telSMN mutation–detection rate for patients with type II or type III SMA can be explained by the presence of these types of mutations. One method for investigation of the possibility of whole-exon deletions or duplications, as well as of mutations that affect splicing, is to look for abnormal SMN transcripts, by use of RT-PCR. Unfortunately, RNA was available from only 1 of the 12 possible SMA compound heterozygotes in whom no telSMN mutation was identified; RT-PCR analysis using primers spanning the SMN gene failed to reveal any abnormally sized transcripts in this individual. Similarly, semiquantitative RT-PCR was used to determine whether this potential compound heterozygote had a reduced amount of full-length SMN transcript in lymphocytes; however, the patient had a significant amount of full-length transcript, suggesting that the pathogenic mechanism for his telSMN mutation (if one is present) is not at the level of transcription.

Type and Distribution of Small telSMN Mutations

To date, a total of 13 small mutations in the telSMN gene have been reported (fig. 1 and table 2), including four frameshift mutations (Bussaglia et al. 1995; Brahe et al. 1996; Parsons et al. 1996, 1998), seven missense mutations (Lefebvre et al. 1995; Bürglen et al. 1996*b*; Hahnen et al. 1997; McAndrew et al. 1997; Rochette et al. 1997; Talbot et al. 1997; this study), and two small deletions in consensus splice donor/acceptor sites (Lefebvre et al. 1995). Although only a relatively small number of intragenic telSMN mutations have been identified, many of them have now been found in more than one SMA patient. All five of the telSMN mutations identified in our SMA patient population have now been found in multiple unrelated individuals; similarly, several other intragenic telSMN mutations have been reported by more than one laboratory (Lefebvre et al. 1995; Hahnen et al. 1997; McAndrew et al. 1997; Rochette et al. 1997; this study). Our data support the possibility of an ancestral origin for the three mutations identified in more than one individual in our patient population; all three patients with the A2G missense mutation were found to have a rare (~3% of normals) 5′ UTR polymorphism on the same allele as that of the mutation (fig. 2). In addition, Ag1-CA microsatellite-marker data also supports the possibility of founder chromosomes for the 800ins11 and 542delGT frameshift mutations, as well as for the A2G missense mutation. In our SMA patients in whom both telSMN mutations have been identified (either individuals homozygously lacking telSMN or compound heterozygotes with a defined intragenic mutation), the allele frequencies for the 800ins11 and A2G mutations are ~1% (4 of 434 chromosomes) and ~0.7% (3 of 434 chromosomes), respectively. Although diagnostic screening could be implemented easily to test for these mutations, as is done routinely for rare cystic fibrosis mutations, the additional requirement of testing for telSMN gene dosage, by quantitative PCR, makes this screening strategy less feasible.

Because most SMA patients are homozygously lacking telSMN, characterization of the relatively rare intragenic telSMN mutations is valuable for identification of important functional domains of the gene. The group of telSMN mutations identified to date strongly suggest that the 3′ end of the gene is essential for telSMN function (fig. 1); except for the A2G missense mutation, all of the nonframeshift mutations identified in telSMN cluster within exons 6 and 7. Recent evidence supports a role for SMN in snRNP biogenesis and function (Liu and Dreyfuss 1996; Fischer et al. 1997; Liu et al. 1997). Immunofluorescence studies using a monoclonal antibody to the SMN protein have revealed that the SMN protein is localized to novel nuclear structures called "gems," which display similarity to and possibly interact with coiled bodies, which are thought to play a role in the processing and metabolism of small nuclear RNAs (snRNAs) (Liu and Dreyfuss 1996). The region of the SMN protein encoded by exon 2 has been shown to be necessary and sufficient for nucleic acid binding (Lorson and Androphy 1998). The SMN protein has been found to be tightly associated with the novel protein SIP1 (SMN-interacting protein 1), within a complex that also contains several spliceosomal snRNP proteins (Liu et al. 1997). In addition, in a Xenopus oocyte model, antibodies directed against this complex have been shown to block both the assembly of the Sm class of snRNP proteins by spliceosomal snRNAs and the nuclear import of the snRNP complex (Fischer et al. 1997), providing additional evidence for the proposed biological role of the SMN protein in snRNP biogenesis and function. The SMN protein has been found to contain two separate binding sites for the Sm proteins and SIP1; the Sm protein binding site is located within the conserved carboxy terminus of the SMN protein (amino acids 240–267), which corresponds to the 5′ end of exon 6, at the DNA level (fig. 1). Three of the intragenic telSMN mutations (P245L, 800ins11, and S262I) identified to date occur within this domain (Parsons et al. 1996; McAndrew et al. 1997; Rochette et al. 1997); however, the other six missense and consensus splice-site mutations occurring in exons 6 and 7 are located farther downstream. A recent study (Lorson et al. 1998) has defined a 30–amino acid region, farther toward the 3′ end of exon 6 (amino acids 249–278), that is essential for oligomerization of the SMN protein (fig. 1). Several of the missense telSMN mutations (S262I, Y272C, T274I, and G279V) were shown to result in extremely inefficient self-association; furthermore, a direct correlation between impairment of oligomerization capability and severity of SMA phenotype was demonstrated (Lor-

son et al. 1998). Except for the novel A2G mutation, all telSMN missense mutations reported to date cluster within or immediately adjacent to this oligomerization domain. The only missense mutation identified outside this region is the A2G mutation, which occurs closely upstream from the SIP1 binding site, at amino acids 13–44 (Liu et al. 1997). It is possible that this substitution sufficiently disrupts the conformation of the SMN protein to alter SIP1 binding; the replacement of alanine by glycine has been shown to have a significant destabilizing effect on peptide α-helical structure (Lyu et al. 1990; Chakrabartty et al. 1991).

Genotype-Phenotype Correlation

Since ~95% of SMA patients are homozygously lacking exon 7 of the telSMN gene, regardless of clinical phenotype (Lefebvre et al. 1995), genotype-phenotype correlation for SMA has not been straightforward. However, additional research has shown that all losses of telSMN exon 7 are not equal; deletions of telSMN are more common in severely affected patients with type I SMA, whereas gene conversions between the telSMN and cenSMN genes are associated with a milder disease phenotype (Burghes 1997; Campbell et al. 1997; DiDonato et al. 1997; McAndrew et al. 1997; Simard et al. 1997). The assignment of specific intragenic telSMN mutations to disease phenotypes is complicated by the effect of these different types of lost telSMN allele. Genotype-phenotype correlation for SMA also has been demonstrated at the protein level: the reduction of SMN protein in patients has been found to be directly correlated with the severity of the SMA phenotype (Coovert et al. 1997; Lefebvre et al. 1997). Within our patient population, frameshift telSMN mutations appear to cause more-severe SMA phenotypes (types I and II) than do missense mutations (types II and III). However, a number of exceptions to this rule have been reported by other groups; the 432del4 frameshift mutation has been found in Spanish SMA patients of all three clinical types (some of them consanguineous), and the missense mutations Y272C and G279V have been found in severely affected patients with type I SMA.

The characterization of these intragenic telSMN mutations has provided additional information regarding the potential role of the cenSMN gene in modifying the SMA phenotype. An increased cenSMN copy number (which can be found as a result of sequence conversion of telSMN to cenSMN) has been reported to be correlated with a milder disease phenotype in patients who are homozygously lacking telSMN (Burghes 1997; Campbell et al. 1997; DiDonato et al. 1997; McAndrew et al. 1997). Analysis of cenSMN copy number in our patients with frameshift mutations demonstrates that this cenSMN gene dosage effect also occurs in SMA compound heterozygotes; with regard to both recurring frameshift mutations (800ins11 and 542delGT), patients with increased cenSMN copy number had significantly less severe SMA phenotypes (table 3). The idea that the cenSMN gene can act in a compensatory manner, to modify the SMA phenotype, is particularly intriguing as a basis for a potential therapeutic strategy; perhaps techniques will be developed to manipulate expression of the cenSMN gene, as a means of replacing an absent or defective telSMN gene.

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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 type I SMA [Werdnig-Hoffmann disease; MIM 253300], type II SMA [MIM 253550], and type III SMA [Kugelberg-Welander disease; MIM 253400])

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