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ARTICLE

# An essential SMN interacting protein (SIP1) is not involved in the phenotypic variability of spinal muscular atrophy (SMA)

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**The survival motor neuron (SMN) protein and the SMN interacting protein 1 (SIP1) are part of a 300 kD protein complex with a crucial role in snRNP biogenesis and pre-mRNA splicing. Both proteins are colocalised in nuclear structures called gems and in the cytoplasm. Approximately 96% of patients with autosomal recessive spinal muscular atrophy (SMA) show mutations in the *SMN1* gene, while about 4% fail to show any mutation, despite a typical SMA phenotype. Additionally, sibs with identical 5q13 homologs and homozygous absence of *SMN1* can show variable phenotypes which suggest that SMA is modified by other, yet unknown factors. Since both genes, *SMN1* and *SIP1*, belong to the same pathway and are part of the same protein complex, it is obvious to ask whether mutations within *SIP1* are responsible for both the phenotypic variability and the appearance of non-SMN mutated SMA patients. First, we identified the chromosomal location of *SIP1* and assigned it to chromosomal region 14q13–q21 by fluorescence *in situ* hybridisation. No SMA related disorder has yet been assigned to this chromosomal region. Next, we determined the exon–intron structure of the *SIP1* gene which encompasses 10 exons and identified five transcription isoforms. We sequenced either RT-PCR products or genomic DNA covering the complete coding region from 23 typical SMA patients who had failed to show any *SMN1* mutation. No mutation and no polymorphism was found within *SIP1*. Additionally, we sequenced RT-PCR products or genomic fragments of the entire *SIP1* coding region from 26 sibs of 11 SMA families with identical genotypes (Δ7SMN/Δ7SMN or Δ7SMN/other mutation) but different phenotypes and again no mutation was found. Finally, we performed quantitative analysis of RT-PCR products from the same 26 sibs. No difference in expression level of the five isoforms among phenotypically variable sibs was observed. Based on these data, we suggest that neither the phenotypic variability nor the 5q-unlinked SMA are caused by mutations within *SIP1*.** *European Journal of Human Genetics* (2000) **8**, 493–499.

**Keywords:** SMN interacting protein 1 (SIP1); survival motor neuron gene (*SMN1*); spinal muscular atrophy (SMA); genomic structure; physical location; molecular analysis; neuromuscular disease; mutation analysis

## Introduction

Autosomal recessive spinal muscular atrophy (SMA) is a neuromuscular disorder with an incidence of 1:6000 to 1:10000.[1,2] SMA is characterised by atrophy and muscle weakness of voluntary muscles. Three types of SMA (Types I–III) have been defined based on age of onset and achieved motor milestones.[3] In most families, there is a high degree of similarity between the clinical picture of affected sibs.[4] Nevertheless, there are quite a few reports in which large phenotypic variability has been described among sibs with identical 5q13 homologs.[5–8] The main SMA determining gene is the survival motor neuron gene (*SMN1*) which in SMA patients is mutated in about 96%.[9–11] Of the 5q13-linked SMA patients, 96.4% show homozygous absence of *SMN1* exons 7 and 8 or exon 7 only, whereas 3.6% present a

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compound heterozygosity with a subtle mutation on one chromosome and a deletion/gene conversion on the other chromosome.[10] The severity of SMA is strongly influenced by *SMN2*, a highly homologous copy of *SMN1*. The more *SMN2* copies a patient has, the milder is the SMA phenotype.[11–14] The explanation for this effect is based on a single nucleotide difference in exon 7 of *SMN1* compared with *SMN2* which leads to alternative splicing and disruption of a putative exonic splicing enhancer.[15] None of the polymorphisms recently reported within intron 6 are responsible for the alternative splicing.[16] Whilst *SMN1* produces only full length transcript, *SMN2* mainly produces alternatively spliced transcript.[9,15,17] Furthermore, it has been shown that exon 7 is part of a domain responsible for the oligomerisation of the SMN protein,[18] In other words, the protein produced by *SMN2* is less efficient and its role can be partially compensated by an increased number of *SMN2* copies.

About 4–5% of typical SMA patients showed by quantitative PCR analysis to have two *SMN1* copies and no evident mutations, suggesting no linkage to 5q.[11]

SMN is part of a 300-kD protein complex, in which SMN interacts with itself, SIP1 (SMN interacting protein 1), Sm proteins that are common components of spliceosomal snRNPs, and other, yet unknown components.[19–21] SMN and SIP1 are co-localized in nuclear structures called gems and in the cytoplasm; both proteins are expressed in similar tissues.[19] The SIP1-cDNA of 1285 bp encodes a protein of 279 amino acids, which binds to a distinct binding site at the N-terminus (aa 13–44) of SMN. This SMN-SIP1 complex has an essential function in spliceosomal snRNP-biogenesis (assembly and recycling of snRNPs) and in pre-mRNA splicing.[20–22] It is therefore obvious to ask whether SIP1 influences the variability of the SMA phenotype or whether in patients who failed to show 5q-linked SMA the disease is caused by mutations within SIP1.

## Materials and methods

### Patient samples

All patients fulfilled the diagnostic criteria for proximal spinal muscular atrophy defined by the International SMA Consortium[3] and Zerres and Rudnik-Schöneborn.[23] According to Zerres and Rudnik-Schöneborn[23] type I SMA patients have an age of onset below 10 months and are never able to sit; type II SMA patients have an age of onset below 18 months and are unable to walk; type IIIa SMA patients have an age of onset $< 3$ years; and type IIIb SMA patients $> 3$ years, the latter showing a mild course with a high probability of remaining ambulant for decades. Informed consent was obtained from all subjects.

Twenty-three typical SMA patients (5 type 1, 2 type II and 16 type III), who did not show mutations in the *SMN1* gene and carried two *SMN1* copies, have been previously described[11] and were now analysed for mutations in the *SIP1* gene. From 14 patients (one type I, one type II and 12 type III) permanent lymphoblastoid cell lines were available for RNA isolation. Additionally, lymphoblastoid cell lines from 26 sibs belonging to 11 families were available for molecular genetic analysis of *SIP1*. In each family sibs show identical haplotypes and identical *SMN1* mutations on both chromosomes. One family has four sibs (two type III SMA and two unaffected sibs); three families have three sibs each (one family with a type IIIa SMA and two unaffected sibs, the second family with a type IIIa and two type IIIb SMA sibs, and the third family with a type II and two type IIIa SMA sibs); and seven families with two sibs each (three families with one type III and one unaffected sib, one family with one type II and one type IIIa SMA sib, and three families with one type IIIa and one type IIIb SMA sib).

### DNA isolation and haplotype analysis

DNA was isolated from blood samples or permanent lymphoblastoid cell lines by the salting-out method.[24] The multicopy polymorphic markers Ag1-CA[25] and C212[26] were used for haplotype analysis as previously described.[27]

### Human BAC library screening

A human BAC library (CEPH human BAC library No. 751, 46XX lymphoblastoid cell line ligated into pBELOBAC) was screened with radioactively labelled full length *SIP1* cDNA. Hybridisation and scoring of positive signals were carried out according to the given recommendations of RZPD Berlin. The obtained BAC clone containing the complete *SIP1* gene has the following reference: B869G2 (CEPHB751M03476Q3).

### Determination of the exon–intron structure of *SIP1* by sequencing BAC B869G2

BAC DNA was purified with the midiplasmid-kit (Qiagen, Hilden, Germany). 800 ng of DNA were used for cycle sequencing which was performed with BigDye Terminator Reaction Kit (PE Applied Biosystems, Weiterstadt, Germany) and primers derived from the *SIP1* cDNA sequence. Sequencing was carried out on the automated ABI 377 A sequencer (Applied Biosystems).

Exon–intron structure was identified by sequence pair comparison between genomic and cDNA sequence of *SIP1* using the BESTFIT program (HUSAR-Heidelberg). Primers were designed from each identified exon in both directions in order to determine the intronic flanking sequences.

### RNA isolation, RT-PCR and cloning of PCR products of *SIP1*

RNA was isolated from Epstein-Barr virus transformed lymphoblastoid cell lines using Trizol kit (Life Technologies, Karlsruhe, Germany). First-strand cDNA synthesis was performed with oligo-dT primers, 4 μg total RNA and M-MLV reverse transcriptase (Life Technologies) according to the manufacturer's instructions. The single-stranded cDNAs were

amplified with 10 ng of each primer SIP1-forward (5'-AGGTGTCTATTAGTGCGCCTG-3') within exon 1 and SIP1-reverse (5'-TGTTCATCAAAAACTGAGTGGG-3') within exon 10, 200 μM each dNTP, 1 U Taq-polymerase (Life Technologies), 1.5 mM $MgCl_2$ within 50 μl reaction volumes. PCR conditions included a 7 min initial denaturation at 94°C followed by 32 cycles of 1 min at 94°C, 1 min at 58°C and 1.5 min at 72°C, with a final extension of 7 min at 72°C.

Since PCR of *SIP1* cDNA revealed four faint fragments next to a strong full-length RT-PCR product, we assumed the existence of various alternatively spliced transcripts. These RT-PCR products were cloned into the pCRII-TOPO cloning vector (Invitrogen). 400 ng of plasmid DNA were used for cycle sequencing.

For mutation analysis RT-PCR products were too faint and too large for direct sequencing. Therefore, 1 μl of a 1:100 dilution of the first PCR product was reamplified with nested PCR primers. The *SIP1* cDNA was subdivided into two pieces, A and B, and amplified with the following primers: fragment A (584 bp) with primers SIP1-nest:-a 5'-CGCTGTGACCTAGAATGG-3' flanking exon 1 and SIP1-nest-b: 5'-TGATTCATTCTGCTAACAATA-3' within exon 6 and fragment B (259 bp) with the primers SIP1-nest-c: 5'-AGATGGCTTTATGCTTTATTG-3' within exon 8 and SIP1-nest-d: 5'-GAGTTAGGCTGCCTTCATG-3' flanking exon 10. PCR conditions were as described for the first PCR reaction except the annealing temperature which was 54°C.

Alternatively spliced exons 6, 7 and 8 were analysed from genomic DNA (see below). Direct sequencing of RT-PCR products including these exons resulted in too high a background.

**PCR of genomic SIP1 exons**

From those patients where no RNA was available each exon was amplified from genomic DNA. Alternatively spliced exons 6–8 were sequenced from genomic DNA from all patients. PCR reaction: 40 ng of genomic DNA was amplified using 10 pmol of each primer (Table 1) in a total volume of 25 μl containing 200 μM dNTPs, 1 U Taq-polymerase (Life Technologies) with 1.5 mM $MgCl_2$. Cycling conditions included a 1 min initial denaturation at 94°C followed by 32 cycles of 45 s at 94°C, 45 s at various temperatures (Table 1) and 45 s at 72°C, with a final extension of 7 min at 72°C. Cycle sequencing was carried out with 60 ng DNA as described above.

***Quantitative analysis of SIP1 transcription isoforms*** cDNAs were amplified in a total volume of 25 μl containing 10 pmol of each primer SIP1-5 forward (5'GCCAAAATCTGAAGATGAAG-3') within exon 5 and SIP1-9rev (5'-AACCAAGCAGATTAATAAATTC-3') within exon 9 200 μM dNTPs, 1 U Taq-polymerase (Life Technologies) with 1.5 mM $MgCl_2$. Cycling conditions include a 7 min initial denaturation at 94°C followed by 25 cycles of 30 s at 94°C, 30 s at 54°C, 30 s at 72°C and a final extension of 7 min at 72°C. The number of cycles was chosen in order to ensure linear amplification of PCR products.

PCR products were resolved on 10% PAA gels and visualised by silver staining.[6] Gels were 3D scanned (BIORAD imaging system) and densitometric measurements were carried out with ONE-Dscan software (MWG, München, Germany). We evaluated the DNA ratio between each alternatively spliced isoform and the full length SIP1 transcript as well as the expression levels of each isoform among patients.

**Table 1** Sequence of the PCR primers used for amplification of SIP1 genomic DNA

| *Exon* | *Primer* | *Sequence* | *Length of PCR-product (bp)* | *Annealing-temp. (°C)* |
|---|---|---|---|---|
| 1 | SIP-Ex1-f<br>SIP-Ex1-r | 5'-CGCTGTGACCTAGAATGG-3'<br>5'-AATAGACCTGAACTCGCAAC-3' | 297 | 56 |
| 2 | SIP-Ex2-f<br>SIP-Ex2-r | 5'-AAGTGGATCGCTTGTTTTAC-3'<br>5'-CGGAGTAGTCAAATCTTACG-3' | 274 | 54 |
| 3 | SIP-Ex3-f<br>SIP-Ex3-r | 5'-CTGAATAAGAGCAGGGTATG-3'<br>5'-CAGAAATAAATCCAACAGGG-3' | 228 | 54 |
| 4 | SIP-Ex4-f<br>SIP-Ex4-r | 5'-AGATGGTTACAATTTTGTGC-3'<br>5'-GCAAGTGAATCTTTAACGAC-3' | 182 | 54 |
| 5 | SIP-Ex5-f<br>SIP-Ex5-r | 5'-GATTCTCCCCATAACTGGC-3'<br>5'-CAGGGTAGAGTTTGCTGACC-3' | 337 | 56 |
| 6 | SIP-Ex6-f<br>SIP-Ex6-r | 5'AGTAGTTTCCGGAATGTCTC-3'<br>5'-AGCATGAGCTCTGGAGTTAG-3' | 500 | 56 |
| 7 | SIP-Ex7-f<br>SIP-Ex7-r | 5'-AGCATGAGCTCTGGAGTTAG-3'<br>5'-GTATATGTGGCCCACAGTTG-3' | 269 | 54 |
| 8 | SIP-Ex8-f<br>SIP-Ex8-r | 5'-AGGCTTTCTTGTCTATACCC-3'<br>5'-AGGCTTTCTTGTCTATACCC-3' | 328 | 56 |
| 9 | SIP-Ex9-f<br>SIP-Ex9-r | 5'-TCTCTGCTTTTTCTTTGAGG-3'<br>5'-GACTGAGGTGGGAGGATTG-3' | 423 | 56 |
| 10 | SIP-Ex10-f<br>SIP-Ex10-r | 5'-TGGCAGTCCTACACATACTC-3'<br>5'-GAGTTAGGCTGCCTTCATG-3' | 453 | 56 |

**Fluorescence *in situ* hybridisation (FISH)**

FISH was carried out according to standard protocols as previously described.[28] The detection of biotinylated probe was performed by a FITC-avidin system leading to light signals.[29] Slides were evaluated on a Zeiss Axioplan fluorescence microscope. Fifteen metaphases were taken into account to determine the localisation.

## Results

**Chromosomal location and genomic structure of *SIP1***

The *SIP1* gene was assigned to the chromosomal region 14q13–q21 by FISH analysis (Figure 1) using BAC clone B869G2 (CEPHB751M03476Q3) which contains the entire *SIP1* gene. The location was further confirmed by using DNA of the chromosome 14 specific somatic cell hybrid GM10479 (kindly provided by Dr N Spurr) for PCR with *SIP1*-specific primers (Table 1).

BAC B869G2 was used to determine the complete exon–intron structure of the *SIP1* gene. Exons were identified by designing primers starting from the 5' and 3' end of the cDNA and comparing the genomic with the cDNA sequence. After identification of the first and last exon, primers were designed from the *SIP1* cDNA sequence flanking these exons in order to determine the sequence of the adjacent exons. By using this strategy we quickly identified the complete exon–intron structure of *SIP1* which encompasses 10 exons (Table 2). The sequences are deposited in Genbank under accession numbers AJ250932–AJ250939.

PCR of *SIP1* cDNA obtained from RNA of EBV-transformed lymphoblastoid cell lines from control individuals revealed, in addition to a strong PCR product representing the full-length transcript, four faint fragments corresponding to alternatively spliced transcripts of exons 6, 7, 8, and 7 and 8, respectively as shown by sequencing of each cloned PCR fragment (Figure 2). An identical transcription pattern was also obtained from RNA of fresh blood (data not shown).

**Molecular genetic analysis of *SIP1* in SMA patients**

About 4–5% of SMA patients with an undistinguishable phenotype from autosomal recessive 5q13-linked SMA do not show any mutation within the *SMN1* gene and carry two *SMN1* copies, suggesting that these patients have 5q13-unlinked SMA.[11]

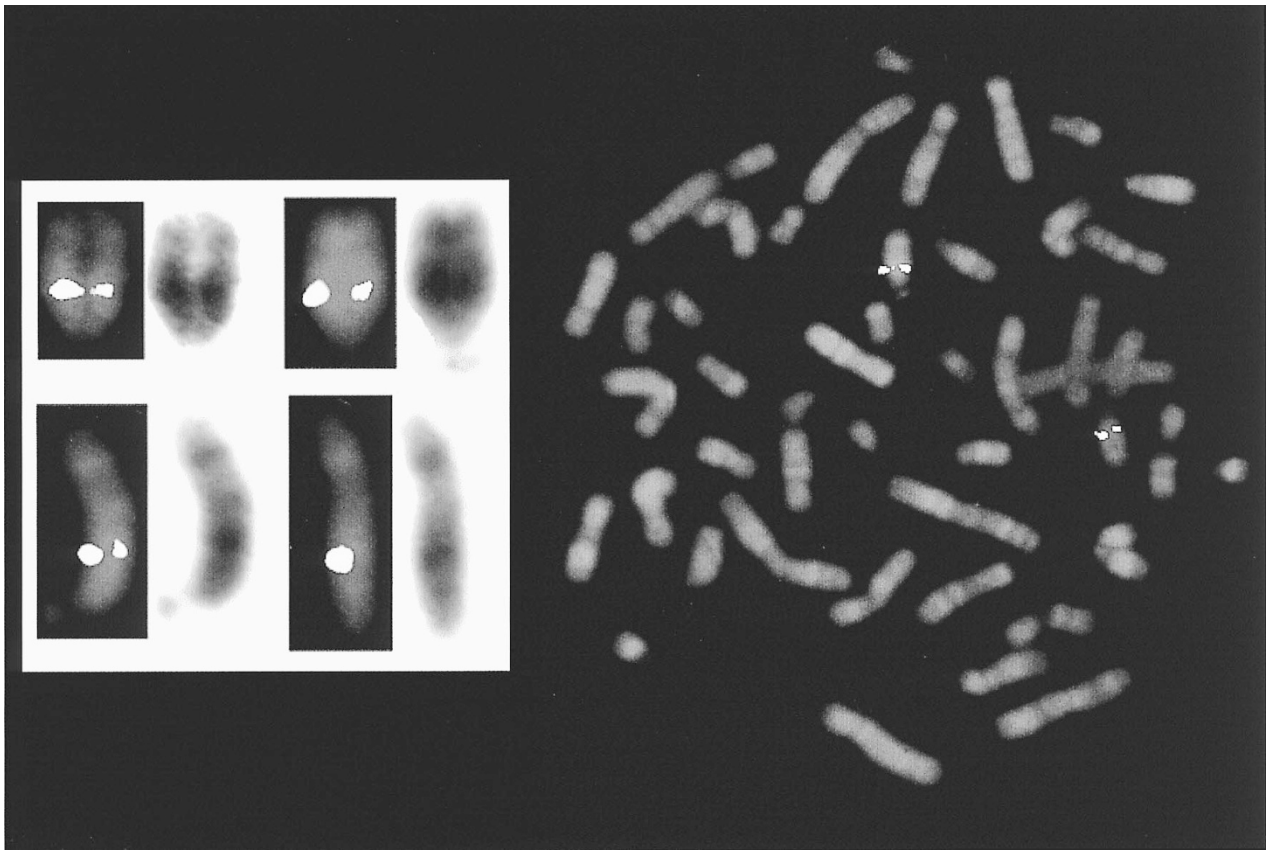

**Figure 1** Metaphase and single selected chromosomes showing result of fluorescence *in situ* hybridisation (FISH) using the specific BAC clone B869G2 which covers the complete *SIP1* gene. *SIP1* was assigned to the chromosomal region 14q13–q12. For comparison the GTG-banding of the chromosomes is also shown. The images were captured with the ISIS digital FISH imaging system (MetaSystems, Altlussheim, Germany) using a XC77 CCD camera with on-chip integration (Sony).

**Table 2** Exon-intron organisation of *SIP1*

| Exon | Exon size (bp) | cDNA position | 5′ splice donor[a] | Intron | 3′ splice acceptor |
|---|---|---|---|---|---|
| 1 | 136 | 117–253 | GGTCCAgtgagt | 1 | ttgtagGATCGA |
| 2 | 85 | 254-338 | ATTTCTgtgagt | 2 | ctctagCTTTCA |
| 3 | 90 | 339-428 | CGACAGgtaagt | 3 | ctttagAATGTG |
| 4 | 59 | 429-487 | GACAATgtgagt | 4 | cttttaGCCAAA |
| 5 | 115 | 488-602 | ATACAAgtaagg | 5 | tttcagATTGGT |
| 6 | 45 | 603-647 | AATCAGgtaaaa | 6 | ttttagGCAACA |
| 7 | 69 | 648-716 | GAATTGgtagta | 7 | ttttagGGAAGA |
| 8 | 111 | 717-827 | TTAGTGgtaagt | 8 | ttttagGATAGC |
| 9 | 59 | 828-886 | TAGCAGgtatag | 9 | tactagGTATTT |
| 10 | 37 | 887-923 | | | |

[a]Exonic sequences are given in capital letters, intronic sequences in small letters.

The *SIP1* gene was analysed in 23 typical SMA patients who carry two *SMN1* copies and no mutation in *SMN1* and were considered 5q-unlinked SMAs. We performed direct sequencing of either RT-PCR products that cover the complete *SIP1* coding region or genomic PCR products that encompass each exon by using intronic flanking primers (Table 1). No mutation and no polymorphism within the coding region or the exon–intron boundaries, and no aberrant transcripts were identified.

Furthermore, we analysed 26 sibs belonging to 11 SMA families who display identical 5q13 haplotypes and identical *SMN1* mutations on both chromosomes but variable phenotypes (unaffected and affected, type II and III or type IIIa and IIIb, respectively, see Materials and Methods) and addressed the question whether *SIP1* could be a modifier gene for SMA phenotypic variability. We looked for DNA sequence variances by sequencing the complete coding region either from RT-PCR products or genomic fragments and for transcription differences by semi-quantitative analysis of RT-PCR products of the 5 isoforms (Figure 3). No mutation, polymorphism or transcription variance among sibs with variable phenotype was identified. We concluded that neither the phenotypic variability nor the 5q13-unlinked SMA is caused by mutations or transcription differences within *SIP1*.

## Discussion

The fact that about 4–5% of SMA patients reveal no mutation within *SMN1* but show SMA-like phenotype on one hand and the intrafamilial variability among *SMN1* detected sibs on the other is rather striking and prompted us to search for the molecular cause of these phenomena. The intrafamilial variability is especially interesting since the milder affected sibs or the unaffected *SMN1* deleted sibs are in most cases females.[5–8,11] The discovery of the modifying factor that prevents these individuals from developing SMA might be of therapeutic relevance.

Since the number of SMA patients showing phenotypic variability or those which are 5q-unlinked is very small, linkage analysis is not suitable. Two strategies can be applied in such cases (a) the candidate approach, eg searching for mutations and expression differences within SMN interacting proteins, which we have chosen in our study, and (b) SMA animal models. Since the knock-out SMN mouse was embryonically lethal[30] and no transgenic SMA animals were available, only the first strategy could be applied. However, during the review process of this paper two groups reported the creation of SMA transgenic mice by introduction of the human *SMN2* gene into the $Smn^{-/-}$ background.[31,32] These will open new possibilities of identifying modifying genes in future. Muscular dystrophies are a good example of similar phenotypes caused by mutations in various interacting proteins belonging to the same pathway and protein complex.[33] By using the second strategy, the animal model, Tsiu's group has successfully identified a modifying gene for CF.[34]

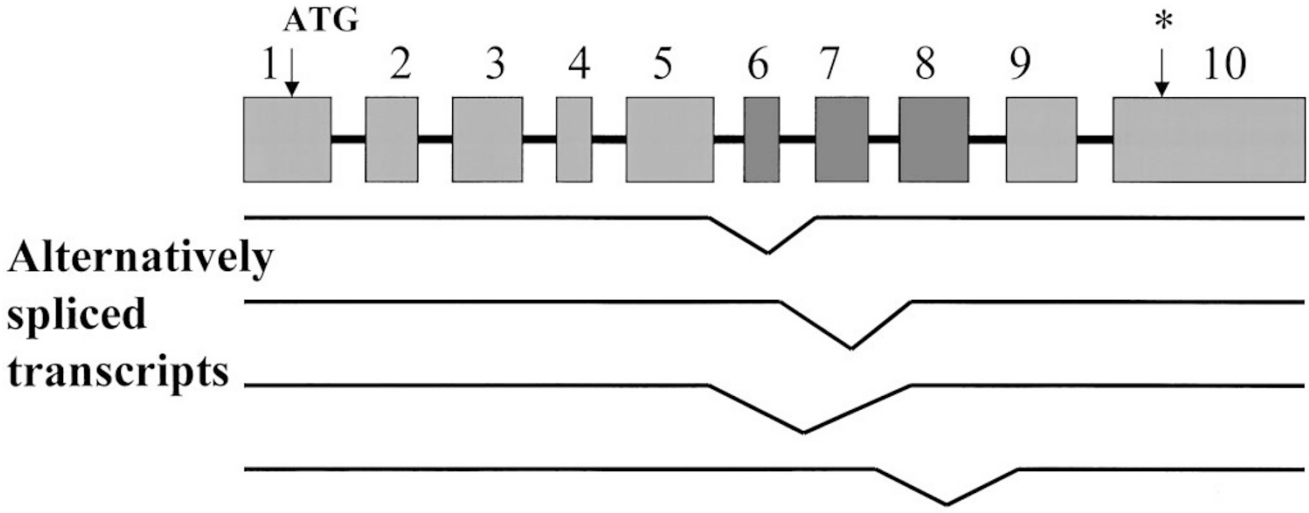


**Figure 2** Schematic representation of the exon–intron structure of *SIP1* and the alternatively spliced transcripts.

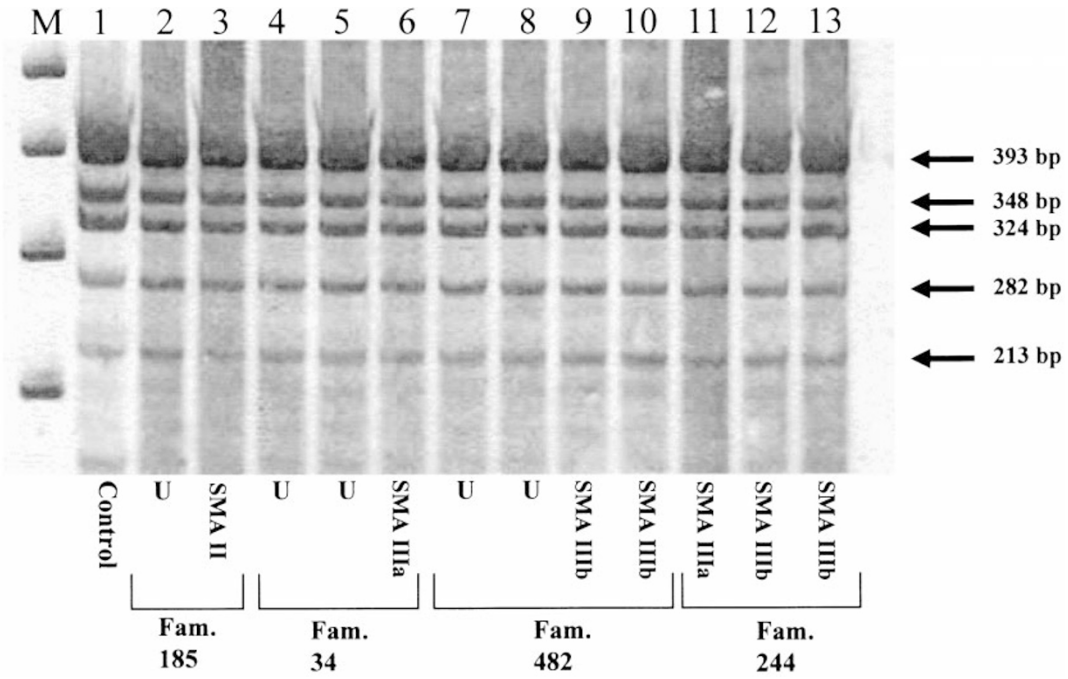


**Figure 3** Quantitative analysis of RT-PCR products of *SIP1* amplified with specific primers localised in exons 5 and 9 from four SMA families with sibs showing all homozygous absence of *SMN1* but variable phenotypes (unaffected and affected or type IIIa and IIIb SMA). The five isoforms are marked with arrows. U = unaffected sib, M = 100 bp ladder (Life Technology).

Although SIP1 and SMN are closely interacting proteins, part of the same protein complex, the same pathway and exhibit the same cellular location in gems and the cytoplasm, our data suggests that SIP1 is neither responsible for 5q-unlinked SMA nor for the phenotypic variability observed in SMA families. Recently a strong interaction between SMN and a neuron specific profilin, PFNII, which is a microfilament-associated protein has been reported.[35] This also suggests that SMN may have other functions than in snRNP biogenesis and pre-mRNA splicing, and that its as yet unknown function may be responsible for the selective degeneration of the motor neurons in the spinal cord. Therefore, other SMN interacting proteins, like PFNII, or RNAs have to be analysed as potential SMA modifiers. Furthermore, the availability of SMA transgenic animals will open new possibilities in future.

## Electronic database information

Accession numbers for SIP1 exons and intronic flanking sequences are from AJ250932 to AJ250939.

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.nih.gov/Omim (for type I SMA [Werdnig–Hoffman disease: MIM 253300], type II SMA [MIM 253550], and type III SMA [Kugelberg–Welander disease: MIM 253400]), SIP1 [MIM602595], SMN1 [MIM600354] and SMN2 [MIM603519]

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