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RESEARCH

Cloning, Characterization, and Copy Number of the Murine Survival Motor Neuron Gene: Homolog of the Spinal Muscular Atrophy-Determining Gene

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Because of a 500-kb inverted duplication, there are two copies of the survival motor neuron (SMN) gene in humans, cenSMN and telSMN. Both genes produce identical ubiquitously expressed transcripts; however, only mutations in telSMN are responsible for spinal muscular atrophy (SMA), the second most common autosomal recessive childhood disease. We have cloned the murine homolog *Smn* and mapped the gene to Chromosome 13 within the conserved syntenic region of human chromosome 5q13. We show that the *Smn* transcript (1.4 kb) is expressed as early as embryonic day 7. In contrast to humans, we found no evidence of alternative splicing. The predicted amino acid sequence between mouse and human SMN is 82% identical, and a putative nuclear localization signal is conserved. FISH data indicate that the duplication of the SMA region observed in humans is not present in the mouse. We also found no evidence of multiple *Smn* genes using Southern blot hybridization and single-strand conformation analysis. Using these methods, we detected at least four copies of *Naip* exon 5 clustering distal to *Smn*. Finally, three biallelic markers were identified within the *Smn* coding region; two are silent polymorphisms, whereas the third changes a cysteine residue to a tyrosine residue in exon 7. Overall, our results indicate that *Smn* is single copy within the mouse genome, which should facilitate gene disruption experiments to create an animal model of SMA.

[The murine *Smn* cDNA sequence has been submitted to GenBank under accession no. U77714. The Mouse Genome Database accession no. for FISH mapping of *Smn* and *Naip* is MGD-INEX-31 and for the genetic mapping of the *Smn* exon 2b microsatellite marker is MGD-CREX-705.]

Proximal spinal muscular atrophy (SMA) is a common autosomal recessive neuropathy. After cystic fibrosis, SMA is the second most frequent monogenic disease of childhood affecting 1 in 10,000 children (Pearn 1980). The most significant clinical finding is proximal, symmetrical limb and trunk muscle weakness, which results from the loss of α-motor neuron cells in the spinal cord. Because SMA is clinically heterogeneous, the disease has been classified into three groups: type I, type II, and type III, based on age at onset and disease severity (Munsat and Davies 1992). Type I SMA is the most severe form and children generally die before 2 years of age.

The gene responsible for SMA is located within a complex genomic region on chromosome 5q11.2–13.3 (Francis et al. 1993, 1995; Thompson et al. 1993; Brahe et al. 1994; Burghes et al. 1994; DiDonato et al. 1994; McLean et al. 1994; Melki et al. 1994; Theodosiou et al. 1994; Daniels et al. 1995; Lefebvre et al. 1995; Roy et al. 1995a,b; Selig et al. 1995; Wang et al. 1995) that contains a 500-kb inverted duplication (Lefebvre et al. 1995). Within this inverted duplication lies the SMA-determining gene, survival motor neuron (SMN). Consequently, SMN is present in two copies, designated cenSMN or

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telSMN, and mutations affecting telSMN are responsible for SMA (Lefebvre et al. 1995). Also located within the SMA region is the neuronal apoptosis inhibitory protein (NAIP) gene (Roy et al. 1995b), which by itself is not a SMA-determining gene. Associated with the intact NAIP gene are several NAIP psuedogenes (ψNAIP) that arose independent of the inverted duplication. Exons 5 and 6 of NAIP are unique to the region and used to distinguish the intact copy of NAIP from ψNAIP copies. These ψNAIP genes are clustered and not interdigitated with the SMN copy genes. cenSMN and telSMN are separated by ~1 Mb of DNA and are virtually identical. They span ~20 kb and consist of nine exons (1, 2a, 2b–8) (Bürglen et al. 1996). There are four differences within the transcribed sequences of cenSMN and telSMN (Lefebvre et al. 1995; Brahe et al. 1996; Hahnen and Wirth 1996). The first DNA variant is at the third position of codon 280 in exon 7 (TTT = cenSMN; TTC = telSMN) and is a silent polymorphism (Lefebvre et al. 1995); the second variant is a g → a substitution of nucleotide 1155 in the cDNA (exon 8) (Lefebvre et al. 1995); and the third and fourth variants are silent polymorphisms (AgC → AgT, codon 28; CAg → CAA, codon 154) in exons 2a and 3, respectively (Brahe et al. 1996; Hahnen and Wirth 1996).

Several groups showed that the absence of at least telSMN exon 7 occurs in the majority of SMA patients (87%–100%) (Chang et al. 1995; Hahnen et al. 1995; Lefebvre et al. 1995; Rodrigues et al. 1995; van der Steege et al. 1995a; Velasco et al. 1996; DiDonato et al. 1997a; Simard et al. 1997). Additionally, the identification of rare point mutations or small deletions in telSMN provides convincing evidence that SMN is the SMA-determining gene (Bussaglia et al. 1995; Lefebvre et al. 1995; Brahe et al. 1996; Parsons et al. 1996).

The SMN copy genes hybridize to a 1.7-kb transcript that is expressed ubiquitously (Lefebvre et al. 1995; van der Steege et al. 1995b). An additional 4.5-kb transcript cross-hybridizing with the SMN cDNA has been reported (van der Steege et al. 1995b). Analysis of alternatively spliced transcripts predict that different SMN isoforms are produced by cenSMN and telSMN (Gennarelli et al. 1995; Lefebvre et al. 1995). Full-length transcripts and transcripts lacking only exon 7 or exons 5 and 7 are products of the cenSMN gene, whereas telSMN produces predominantly full-length transcripts but also those lacking exon 5 (Gennarelli et al. 1995).

Recently, Liu and Dreyfuss (1996) demonstrated that the 40-kD SMN protein is conserved in vertebrates and interacts with the RGG box region of heterogenous nuclear ribonucleoprotein (hnRNP) U, fibrillarin, itself, and several other novel proteins. Immunolocalization studies in HeLa cells using monoclonal antibodies raised against SMN indicated that SMN is part of a novel nuclear structure termed gems, for Gemini of coiled bodies. These observations raise the possibility that SMN may have a function in RNA metabolism.

To decipher the function of the telSMN protein and its role in SMA etiology, we are using the mouse as a model system. In this paper we describe the first step toward this goal, the cloning and preliminary characterization of *Smn*, the murine homolog of the SMA-determining gene. We also present the results from a series of experiments showing that *Smn* is present as a single copy within the mouse genome. This will allow us to characterize the *Smn* protein more easily and determine its function in normal and disease states. In addition, one can begin to study the differences between cenSMN and telSMN and determine why only mutations in telSMN cause SMA.

RESULTS

Cloning and Sequence Analysis of the *Smn* cDNA

Smn cDNA clones were isolated from mouse pre-B cell and 7.5-day whole embryo λgt10 libraries. Additional clones were identified in the expressed sequence tag (EST) database. Six different cDNA clones were subjected to further analysis. All were partial or full-length cDNAs that were similar to human SMN (Lefebvre et al. 1995). The longest clone contained a 1250-bp insert comprising the complete coding and 3′-untranslated region (UTR) (Fig. 1). Sequence analysis of exon 7 revealed that all clones contained a cytosine at position 825, which is the same nucleotide identified at the corresponding position (nucleotide 840) of the human telSMN copy gene (Lefebvre et al. 1995). The overall nucleotide identity between mouse *Smn* and human SMN was 83% across the open reading frame. No sequence conservation was observed in exon 8, which contains the 3′ UTR.

The *Smn* open reading frame encodes a protein of 288 amino acids with a calculated molecular mass of 31.3 kD. Murine *Smn* is slightly smaller than its human counterpart, as three amino acids in exon 1, two in exon 4, and one in exon 7 are absent (Fig. 2A). The predicted mouse protein is 82% identical to human SMN. There are regions that are highly conserved such as the putative 4 amino acid nuclear

localization signal (NLS) in exon 2b. Also conserved are the amino acids encoded by exon 2a and the polyproline stretches in exons 4, 5, and 6, whereas other regions have diverged significantly. Figure 2B summarizes the estimated nucleotide and amino acid identities between murine and human SMN for each exon. BLASTp searches of the publicly available databases using full-length *Smn* protein identified its human homolog, a *Caenorhabditis elegans* protein ($P = 3.9 \times 10^{-15}$), and a hypothetical protein in *Schizosaccharomyces pombe* ($P = 0.011$). The 197 amino acid *C. elegans* protein (Z81048) is located in chromosome III and has no known function. It shares high identity with regions of the *Smn* protein located in exons 2a, 3, and 6. The putative protein was actually identified using GENE FINDER from sequence obtained through the *C. elegans* genome sequencing project. To date, searches with this protein sequence in the *C. elegans* EST database have not yielded any positive clones. Additionally, a transgenic strain using the cosmid that contains this putative protein has not yet been produced. *Smn* also identified a hypothetical 17.4-kD protein in *S. pombe* (Z54354; $P = 0.011$). Normally, this would not be considered a very significant result; however, the same regions of *Smn* exons 2a and 6 were identified. Using the *C. elegans* protein in a BLASTp search, the 17.4-kD protein was identified ($P = 5.9 \times 10^{-5}$) and the area of similarity between the two proteins corresponding to *Smn* exons 2a and 6 was extended.

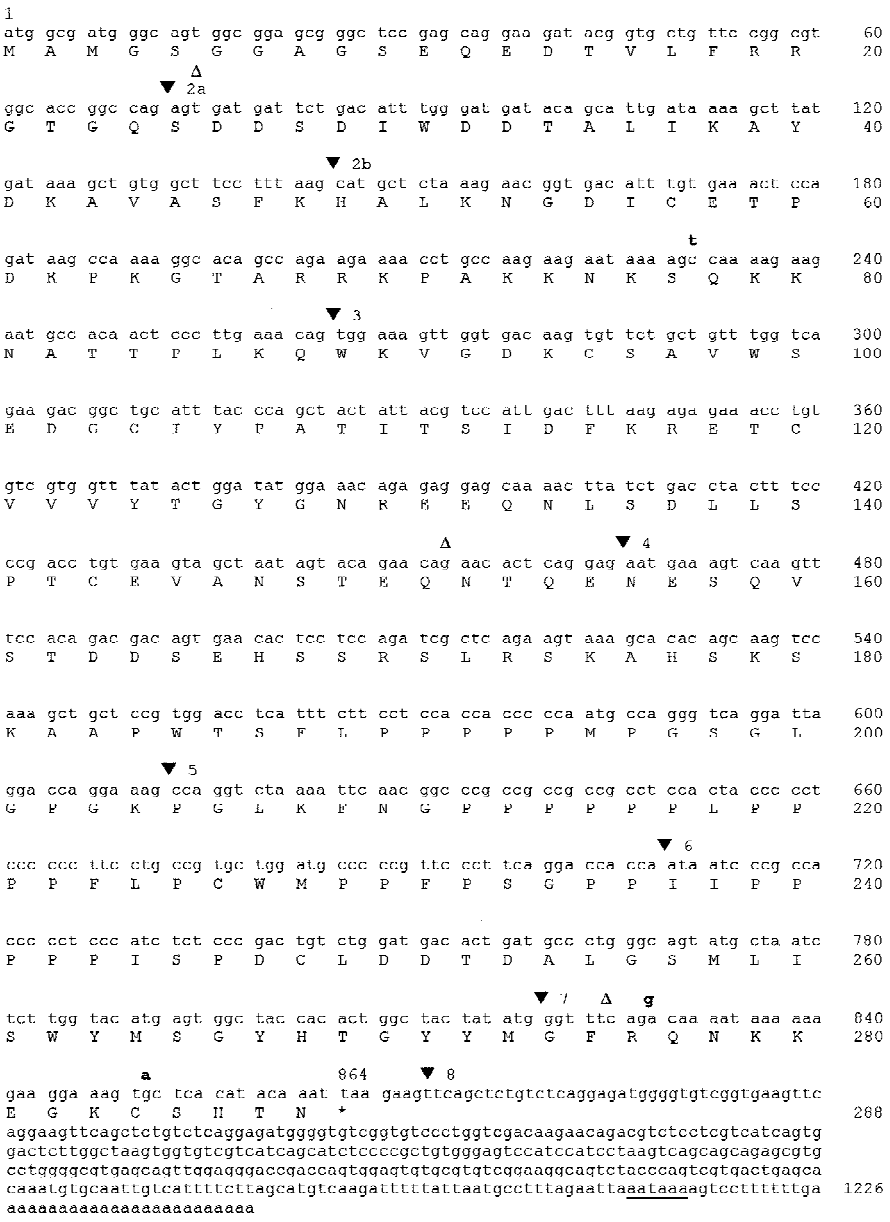


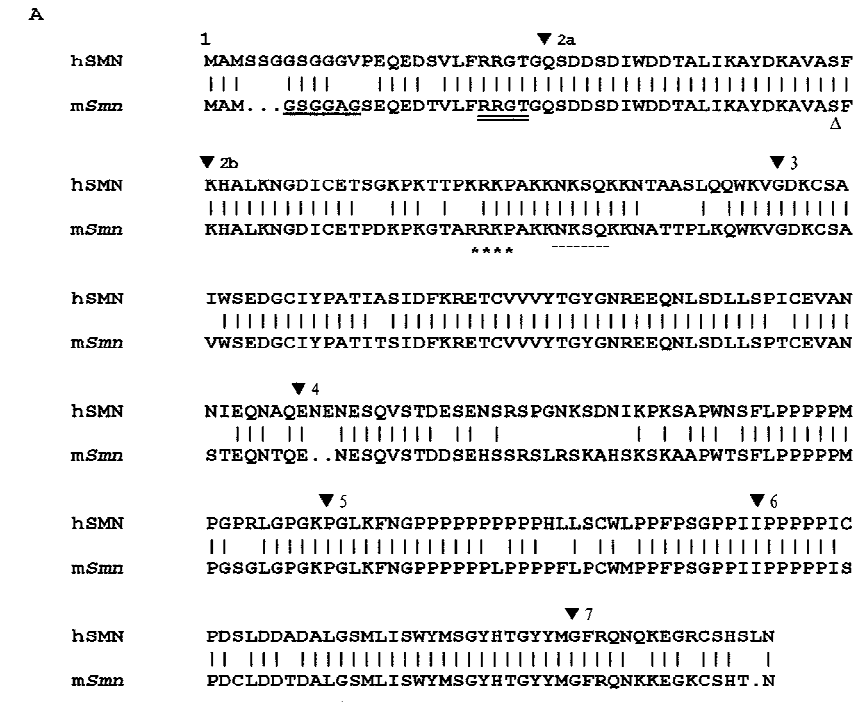
Figure 1 Nucleotide and deduced amino acid sequence of the *Smn* cDNA. The deduced amino acid sequence beginning with the initiating methionine is given. Numbering of nucleotides and amino acids is shown at the right. (▼) Positions of introns; a potential polyadenylation signal (ATAAA) in the 3′ UTR is underlined; (△) the position of the DNA variants in exons 2a, 3, and 7 of human SMN. The polymorphic variants identified in SPRET/Ei DNA in exons 2b and 7 are indicated above the *Smn* sequence. Several other variants were also identified in exon 8 but are not shown because of multiple-base changes. The entire *Smn* cDNA sequence has been confirmed by comparison with the sequence of genomic DNA from the 129/SvJ strain. The GenBank accession no. is U77714.

Characterization of the *Smn* Transcript

A Northern blot containing poly(A)$^+$ mRNA from fetal and adult mouse tissues was used to determine the size, as well as the temporal and spatial distribution, of *Smn* transcripts. A *Smn* cDNA probe that contained ex-

ons 2b–8 hybridized to a full-length transcript of ~1.4 kb in all tissues examined and a 4.4-kb transcript in liver (Fig. 3). Hybridization to fetal tissues indicated that the gene was expressed in the 7-day embryo, the earliest stage analyzed.

To determine whether the full-length transcript is alternatively spliced, *Smn* cDNA from brain, kidney, liver, and spinal cord was used in RT–PCR (see Methods). In all tissues examined, only full-length *Smn* PCR products were identified (data not shown) even after transfer and Southern blot hybridization with *Smn* cDNA as probe.



B

Exon	% nucleotide identity	% amino acid identity
1	74	74
2a	94	100
2b	85	75
3	88	91
4	75	63
5	81	84
6	82	91
7	81	75
8	--	--

Figure 2 (*A*) Amino acid comparison of mouse *Smn* and human SMN. Vertical lines indicate amino acid identity; dots represent gaps that have been introduced to maximize identity. A few possible post-translational modification sites that are conserved between the two species are shown: (Broken underline) Asn glycosylation; (single underline) myristalation; (double underline) cAMP phosphorylation; (△) PKC phosphorylation; and (*) NLS sequence. (*B*) Comparison of percent nucleotide and amino acid identity of murine and human SMN.

Single-Strand Conformation Analysis

In humans, single-strand conformation analysis (SSCA) of exons 7 and 8 was used to distinguish cenSMN from telSMN (Lefebvre et al. 1995). To identify sequence polymorphisms that might be indicative of gene duplication in the mouse, we performed SSCA for each exon of the *Smn* gene. Table 1 lists the primer sets, annealing temperatures, magnesium concentrations, and amplicon sizes for each exon. Two different electrophoretic conditions were used to analyze PCR products from 129/SvJ genomic DNA and 129/SvJ genomic subclones (data not shown). The migration pattern of 129/SvJ DNAs for each of the exons analyzed was identical; however, interstrain polymorphisms were observed in exons 2b, 7, and 8 in SPRET/Ei versus 129/SvJ and C57BL/6JEi DNA. Three of these biallelic polymorphisms are within the *Smn* coding region (exons 2b and 7), and all three strains of mice were homozygous for each SSCA variant (Fig. 1). Figure 4 presents the results obtained for exon 2b, which describes two polymorphisms in SPRET/Ei versus 129/SvJ and C57BL/6JEi. The first is a C/T silent polymorphism at nucleotide 231 of the cDNA that was confirmed by direct sequencing as well as sequence from subcloned SPRET/Ei DNA. The second is an $(A)_{19}$ repeat in SPRET/Ei compared to a tetranucleotide $(ATTT)_6$ repeat in 129/SvJ and C57BL/6JEi. The second polymorphism was used to map the *Smn* gene.

Interspecific Backcross Mapping of *Smn*

The Jackson Laboratory BSS mapping panel [(C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei)], which has now been typed for >2060 loci (Rowe et al. 1994), was used to determine the chromosomal location of the *Smn* gene. The $(ATTT)_6/(A)_{19}$ repeat polymorphism in exon 2b was used to

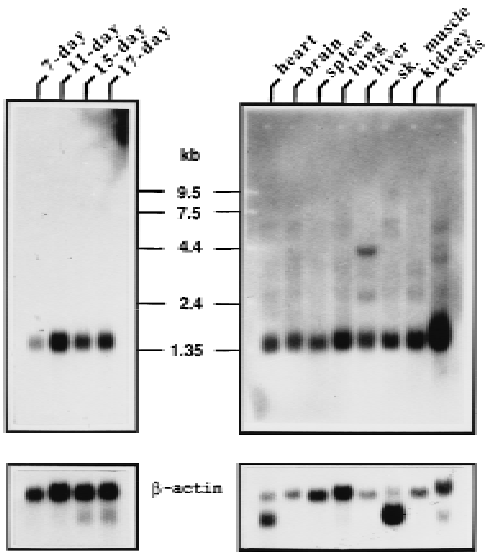


Figure 3 Northern blot hybridization of fetal and adult poly(A)$^+$ mRNA with a partial *Smn* cDNA. *Smn* transcripts (~1.4 kb) are expressed in all tissues and developmental stages analyzed. A 4.4-kb transcript is also present in liver. After hybridization with *Smn*, Northern blots were stripped and reprobed with β-actin to determine the relative amount of poly(A)$^+$ mRNA in each lane.

genotype 94 backcross DNAs. As shown in Figure 5, the segregation of alleles indicated that *Smn* is located on Chromosome 13 and maps between *D13Bir19* and *Efta*. *Smn* cosegregates with 20 other loci (data not shown) on the BSS mapping panel, including *D13Lsd1*, which marks the location of the *Naip* gene (DiDonato et al. 1997b), and *D13Mit37*. The order of a subset of these loci and their genetic distance in centimorgans is provided in Figure 5A.

Contiguous Arrays of Bacterial and Yeast Artificial Chromosomes

Genomic clones corresponding to the mouse SMA region were isolated by screening high-density filters of a mouse 129/SvJ BAC library (Genome Systems) with human SMN exons 6–8 and human NAIP exon 5. DNA pools from a 129/SvJ bacterial artificial chromosome (BAC) library (Research Genetics) were screened using a primer set that amplified *Smn* exon 2b. After it was determined that *D13Mit146* was present in several *Smn* BAC clones and that it was located proximal of *Smn*, BAC DNA pools were rescreened via PCR using *D13Mit146* to extend the contig proximally. Overall, these experiments identified six *Naip*, six *Smn*, and one *D13Mit146* positive BAC clones. Table 2 shows the sequence-tagged site (STS) content for the minimal number of BACs required to span the region from *D13Mit146* to *D13Mit37/Naip* exon 5. All BACs were also analyzed by restriction digest and Southern blot hybridization using full length *Smn* cDNA and *Naip* exon 5 as probes. We found no evidence for multiple copies of *Smn* or gene rearrangements within the clones when compared to genomic DNA (data not shown). However, *Eco*RI restriction digests of genomic DNA and BAC clones containing *Naip* exon 5 revealed four distinct copies of *Naip* exon 5, that is, 4 unique fragments in genomic DNA. The largest *Eco*RI fragment (~10 kb) appeared to be present in two copies. All copies can be accounted for in the various BAC clones (data not shown). Confirmation of these results is provided in the recently published paper by Scharf et al. (1996).

We also identified six yeast artificial chromosomes (YACs) from the MIT database that were positive for markers that cosegregrated with *Smn* and *Naip* on the BSS genetic map. This YAC contig, which is ~1.1 cM in length, contains the region from *D13Mit36* to *D13Mit70* and was analyzed for the presence or absence of STSs and simple-sequence length polymorphisms (SSLPs) via PCR (Table 2). The SSLP marker *D13Mit146* was positive in YAC clones: 187D4, 380D1, and 334g7 and in BAC clone 321I22 but was negative in all other YACs and BACs tested. This indicated that *D13Mit146* maps distal to *D13Mit36* and proximal to *Smn* and *D13Lsd1/Naip*. The *Smn* gene STSs were positive in YACs 187D4, 380D1, and 334g7 and BACs 20g19 and 227n6. The combined BAC and YAC data indicate that if *Smn* is duplicated, both copy genes would have to map distal to *D13Mit146* and proximal of *D13Lsd1* (~180 kb interval). This result is completely inconsistent with its human homolog where cenSMN and telSMN are separated by ~1 Mb (Lefebvre et al. 1995). Figure 6 presents a physical map of the region surrounding *Smn* based on STS content.

Fluorescence in Situ Hybridization Mapping

The 500-kb inverted duplication of the SMA region in humans gave rise to two copies of SMN separated by ~1 Mb (Lefebvre et al. 1995). We have used fluorescence in situ hybridization (FISH) on mouse metaphase and interphase cells to determine whether this duplication also exists in the mouse and to confirm the localization of *Smn* in Chromosome 13. A 100-kb *Naip* BAC (152P21) that contained one copy of *Naip* exon 5 and a 180-kb *Smn*

Table 1. Novel STSs Used for SSCA and STS Content Mapping

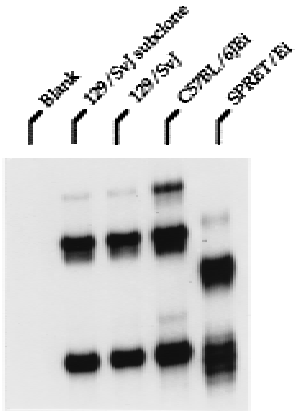
Locus	Forward primer (5' → 3')	Reverse primer (5' → 3')	Final [Mg^{2+}]	Temp. (°C)	Size (bp)
Smn 1	ATg gCg ATg ggC AgT ggC	CTg gCC ggT gCC ACg CCg	1.5	54	72
Smn 2a	TTA AgC ACA TCT ATC TAT AAC	gTA TgA CCT gAg gAA ATA Agg AAA	3.0	55	164
Smn 2b	CCT CCA TgA CAC ATA gAT CT	CAg gAC CCT ggT TAA ATC A	2.5	56	206
Smn 3	Tgg AAA gTT ggT gAC AAg Tg	gCT CCT CTC TgT TTC ATC CA	3.0	55	201
Smn 4	AAT gAA AgT CAA gTT TCC ACA	CTg gAA AAC TTT CAT AgA Agg	3.0	56	173
Smn 5	AgA TAC TAA CTT ggA AAT ATT C	gTg TAA CCT CAg CTC TTg	3.0	56	152
Smn 6	ATA ATC CCg CCA CCC CCT CCC	AAC TgC AAA gAg CAT gTC Tgg	3.0	56	153
Smn 7	TCC AgC Cgg gCT TgA ATT	AAT TAT ACA AAA ggT AAA ATT AgC	5.0	56	172
Smn 8	gTT CAg CTC TgT CTC Agg Ag	gCA CAT TTg TgC TCA gTC ACg	5.0	56	242

BAC (20g19) were used as probes in FISH. The results indicated that these genes colocalized to mouse Chromosome 13 (Fig. 7). From three independent experiments, >30 metaphase and 50 interphase cells were evaluated. In all metaphase cells, signals from *Smn* and *Naip* probes were seen clearly on the two chromatids in the region MMU13D1-2.1 in 90% of cells. Dual-color FISH experiments indicated that *Smn* and *Naip* signals were close together or overlapping with each other, in random order, in the majority of cells. Only a single copy per chromosome was detected in all of the metaphase and interphase cells analyzed. Thus, the 500-kb inverted duplication observed in humans is not present in mouse. Furthermore, from the interphase study, the *Smn* and *Naip* genes are separated by >50 kb.

DISCUSSION

In this paper we present the cloning and characterization of the murine homolog of the human survival motor neuron gene. The *Smn* open reading frame (ORF) is 864 bp long and encodes a protein of 288 amino acids with a molecular mass of 31.3 kD. Sequence comparison across the ORF at the nucleotide level indicates that the gene is 83% identical to its human counterpart, but there is no conservation in the 3′UTR (exon 8).

Comparison of the amino acid sequence for murine and human SMN showed 82% identity, suggesting that both proteins have a similar tertiary structure. There is also a 4 amino acid nuclear localization signal (NLS) in exon 2b that is conserved but was not reported in the original cloning of human SMN (Lefebvre et al. 1995). This would explain the staining of gems within the nucleus reported by Liu and coworkers using monoclonal antibodies raised against SMN (Liu and Dreyfuss 1996). Further studies are necessary to determine whether this putative NLS is functional or whether a novel NLS is present within the *Smn* protein that directs it to the nucleus. A polymorphism that results in an amino acid substitution (CYS → TYR) at codon 284 in exon 7 of SPRET/Ei mice was identified, which suggests that this cysteine is not crucial to the tertiary and quaternary structure of the protein. Thus, if a missense mutation is identified at this position in humans, one would predict it to be a tolerable substitution, but the phenotypic consequence will most likely depend on the state of the other telSMN gene. Additional comparison of the two predicted proteins indicated that exons 2a and 6 and the polyproline stretches may be functionally significant given their high degree of conservation. Protein alignment also revealed areas of divergence such as exon 4, indicating a lack of selective pressure on this portion of the protein. Finally, a BLASTp search of the publicly available databases using full-length *Smn* protein identified its human homolog, a *C. elegans* protein of unknown function in chromosome III and a 17.4-kD protein in *S. pombe*. These proteins, especially the *C. elegans* protein, share a high degree of identity to portions of *Smn* exons 2a, 3, and 6, which indicates an evolutionarily conserved function for these regions. Several point mutations have been reported within telSMN, but none are within these conserved areas (Bussaglia et al. 1995; Lefebvre et al. 1995; Brahe et al. 1996; Parsons et al. 1996). It

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129/SvJ      cctccatgac acatagatct tatttattta tttatttatt tatttactgt tttcatagCA TGCTCTAAAG   70
C57Bl/6JEi   ---------- ---------- ---------- ---------- ---------- ---------- ----------
SPRET/Ei     ---------- ---------t tttttttttt tttttttt        ----- ---------- ----------

129/SvJ      AACGGTGACA TTTGTGAAAC TCCAGATAAG CCAAAAGGCA CAGCCAGAAG AAAACCTGCC AAGAAGAATA  140
C57Bl/6JEi   ---------- ---------- ---------- ---------- ---------- ---------- ----------
SPRET/Ei     ---------- ---------- ---------- ---------- ---------- ---------- ----------

129/SvJ      AAAGCCAAAA GAAGAATGCC ACAACTCCCT TGAAACAGgt gatagattga tttaaccagg gtcctg       206
C57Bl/6JEi   ---------- ---------- ---------- ---------- ---------- ---------- ------
SPRET/Ei     ----T----- ---------- ---------- ---------- ---------- ---------- ------
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Figure 4 Polymorphism in *Smn* exon 2b identified by SSCA. An interstrain polymorphism between SPRET/Ei and 129/SvJ or C57BL/6JEi was identified. Sequence analysis identified a tetranucleotide repeat $(AAAT)_6$ in 129/SvJ and C57BL/6JEi compared to an adenine repeat $(A)_{19}$ in SPRET/Ei. Additionally, a silent polymorphism in SPRET/Ei DNA was also identified. This is a C/T transversion, and both triplets code for the same amino acid (serine).

would be interesting to identify and determine the deleterious effects, if any, that missense mutations might have within these regions of conservation.

Analysis of *Smn* expression by Northern blot hybridization identified a ubiquitiously expressed 1.4-kb transcript and a 4.4-kb transcript specifically expressed in liver. We show that *Smn* is expressed prenatally as early as embryonic day 7 in the mouse. *Smn* differs from the human 1.7-kb transcript (Lefebvre et al. 1995; van der Steege et al. 1995b) by 300 bp, which can be accounted for by the smaller 3′ UTR in the mouse transcript. The 4.4-kb liver-specific transcript could be the same as the 4.5-kb transcript seen by van der Steege et al. (1995b). RT–PCR indicated that *Smn* is not alternatively spliced in brain, kidney, liver, or spinal cord. This result contrasts that found in humans, where it has been shown that in muscle and lymphoblasts, cenSMN undergoes alternative splicing of exons 5 and 7 in various combinations (Gennarelli et al. 1995; Lefebvre et al. 1995). Although the major transcript of telSMN is full-length, mRNAs lacking exon 5 are also produced (Gennarelli et al. 1995). The absence of alternative splicing in the mouse suggests that only the full-length *Smn* product is important for normal α-motor neuron function. If this is true, then only the full-length SMN protein plays a role in SMA etiology, the variant isoforms having no effect. This hypothesis seems plausible given the fact that telSMN, the gene disrupted in all 5q SMA patients, predominantly produces the full-length SMN transcript. However, in humans, the possibility that cenSMN may modify disease severity cannot be ruled out completely, that is, cenSMN copy number and sequence conversion events must be considered (Velasco et al. 1996; Hahnen et al. 1996; van der Steege et al. 1996; DiDonato et al. 1997a). Ultimately, proof for or against this hypothesis will have to await the production of transgenic animals that contain cenSMN or telSMN. One would expect that crossing a telSMN transgenic mouse to a knockout *Smn* mouse would rescue the phenotype while crosses with cenSMN transgenics would not, as the major transcript produced by cenSMN is an isoform lacking exon 7.

Four methods were used to analyze *Smn* copy

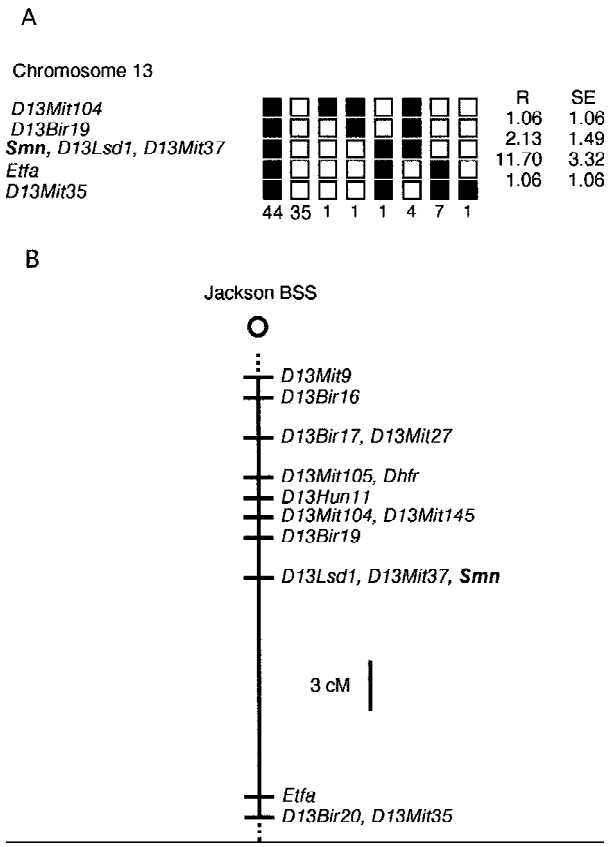

Figure 5 Mapping of *Smn* to Chromosome 13. (*A*) Haplotypes from the Jackson Laboratory BSS backcross panel showing part of Chromosome 13 with loci linked to *Smn*. Loci are listed in order with the most proximal at the top. (■) The C57BL/6JEi allele; (□) the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The estimated percent recombination (R) between adjacent loci is given at *right*, with the standard error (SE) of the estimate. Missing typing for markers *D13Bir17*, *D13Mit105*, *Dhfr*, *D13Mit145*, and *D13Bir19* was inferred from the genotypes of the flanking loci where assignment was unambiguous. The Mouse Genome Database (MGD) accession number for genetic mapping of *Smn* is MGD-CREX-705. (*B*) Map showing part of Chromosome 13 from The Jackson BSS backcross panel. The map is depicted with the centromere toward the *top*. A 3-cM scale bar is shown at *right*. Loci mapping to the same position are listed in alphabetical order. These data can be obtained from The Jackson Laboratory on World Wide Web site http//www.jax.org/resources/documents/cmdata.

number within the mouse: linkage analysis, SSCA, FISH, and physical mapping. The exon 2b $(A)_{19}$/$(ATTT)_6$ polymorphism was used to map the *Smn* gene by linkage analysis to Chromosome 13. There were no ambiguities in the segregation analysis of backcross DNAs. This is in agreement with Scharf et al. (1996), who used a polymorphism in intron 1 to map *Smn* on the same panel of backcross DNAs. The results of SSCA indicated that there were no detectable differences between amplified fragments from 129/SvJ genomic DNA and 129/SvJ genomic subclones that contained each of the exons. However, interstrain polymorphisms in exons 2b, 7, and 8 were detected, and in all instances, mice were homozygous for the intragenic interstrain polymorphisms. Furthermore, as there is very little sequence divergence between human cenSMN and telSMN across the coding and noncoding regions (Lefebvre et al. 1995; Bürglen et al. 1996; C. DiDonato and L. Simard, unpubl.) when compared to murine *Smn*, we postulate that the duplication that gave rise to two SMN genes arose after the divergence of lineages leading to humans and mice. This hypothesis is consistent with our FISH results, where we demonstrated that the large inverted duplication of the human SMA region is not present in mouse. Finally, we determined the physical structure of the *Smn* region by creating a YAC and BAC contig that was analyzed by STS content and Southern blot hybridization. Overall, our physical map of *Smn* was in agreement with our genetic localization. We found no evidence of gene duplication by Southern blot hybridization. Additionally, STS content mapping was able to resolve several loci that cosegregated genetically and allowed us to determine a relative order of markers from centromere to telomere [cen-*D13Mit36–D13Mit146–Smn5′–Smn3′–D13Lsd1–*(*D13Mit37, Naip exon 5*)*–D13Mit203–D13Mit195–D13Mit30–D13Mit70–D13Mit71*–tel]. Although we detected multiple copies of *Naip* exon 5 and *D13Mit37*, we have not ordered them within our contig; therefore, we have placed *D13Mit37* and *Naip exon 5* within parentheses. Proof that multiple copies of *Naip* exon 5 and *D13Mit37* exist is also provided by Scharf et al. (1996), who recently published a physical map of the *Lgn1* critical region. It is difficult to compare our physical map with that of Scharf et al. (1996) as we only have two YAC clones in common. However, the maps are consistent with each other in that both position *Smn* to the same location and the same orientation and that multiple copies of *Naip* exist. Scharf and coworkers detected different *Naip* exon 5 copies by SSCA, whereas we used *Eco*RI restriction digests and Southern blot hybridization with *Naip* exon 5 as probe. In both maps, all *Naip* copies cluster in a region distal to *Smn*. The clustering of all copies of *Naip* is consistent with our FISH results, where a biotinylated (green)

Table 2. STS Content of BACs and YACs Located Between ***D13Mit36*** and ***D13Mit70***

Clone name	Company	YAC (Y); BAC (B)	Size (~ kb)	primary positive	*D13MIT 36*	*D13MIT146*	*Smn*	*D13Lsd1*	*Naip ex 5*	*D13MIT37*	*D13Mit203*	*D13MIT 195*	*D13MIT 30*	*D13MIT 72*	*D13Mit 70*
321l22	R.G	B	170	*Mit 146*		■									
20g19	G.S.	B	180	SMN 6-8		■	■								
152p21	G.S.	B	100	NAIP ex5				■	■	■					
227n6	G.S.	B	130	NAIP ex5			■	■	■	■					
187 D4	R.G.	Y	NA	MIT D.B.	■	■	■	■	■	■					
380 D1	G.S.	Y	NA	MIT D.B.		■	■	■	■	■					
334 g7	G.S.	Y	NA	MIT D.B.		■	■	■	■	■	■	■			
133F10	R.G.	Y	NA	MIT D.B.								■	■	■	
462 H2	R.G.	Y	NA	MIT D.B.									■	■	■
141 E3	R.G.	Y	NA	MIT D.B.											■

For each of the STSs indicated, PCR amplifications were performed in triplicate using single-colony picks or DNA prepared from single colonies of the indicated clone. (■) Positives; (□) negatives. BAC clones were analyzed by restriction mapping and Southern blot hybridization using a variety of probes (data not shown). The *Smn* STS marker represents all *Smn* primer sets listed in Table 1. *Naip* exon 5 and *D13Mit37* are represented multiple times in the region (data not shown) but listed only once for simplicity. (G.S.) Genome Systems; (R.G.) Research Genetics; (NA) not analyzed; (MIT D.B.) MIT database.

signal was confined to a single region on Chromosome 13.

In conclusion, the results reported here integrate the genetic and physical maps of Chromosome MMU13D1-2.1 and confirm that *Smn* and *Naip* are part of the large conserved linkage group with human chromosome 5. This report also provides information and resources that will be useful for positionally cloning other genes in this region including *Lgn1* (Beckers et al. 1995; Dietrich et al. 1995; Scharf et al. 1996). Most importantly, we have presented our findings on the cloning and characterization of *Smn*, the murine homolog of the SMA-determining gene. The fact that *Smn* is single copy will allow us to characterize the *Smn* protein, decipher its normal function, and produce mice deficient for this gene more easily. Such experiments will provide critical information regarding the role of telSMN in the etiology of proximal SMA and why an almost identical copy gene, cenSMN, cannot compensate completely for the absence of telSMN protein.

METHODS

Isolation of *Smn* cDNA Clones

Approximately 1 million phage plaques of a λgt10 pre-B cell

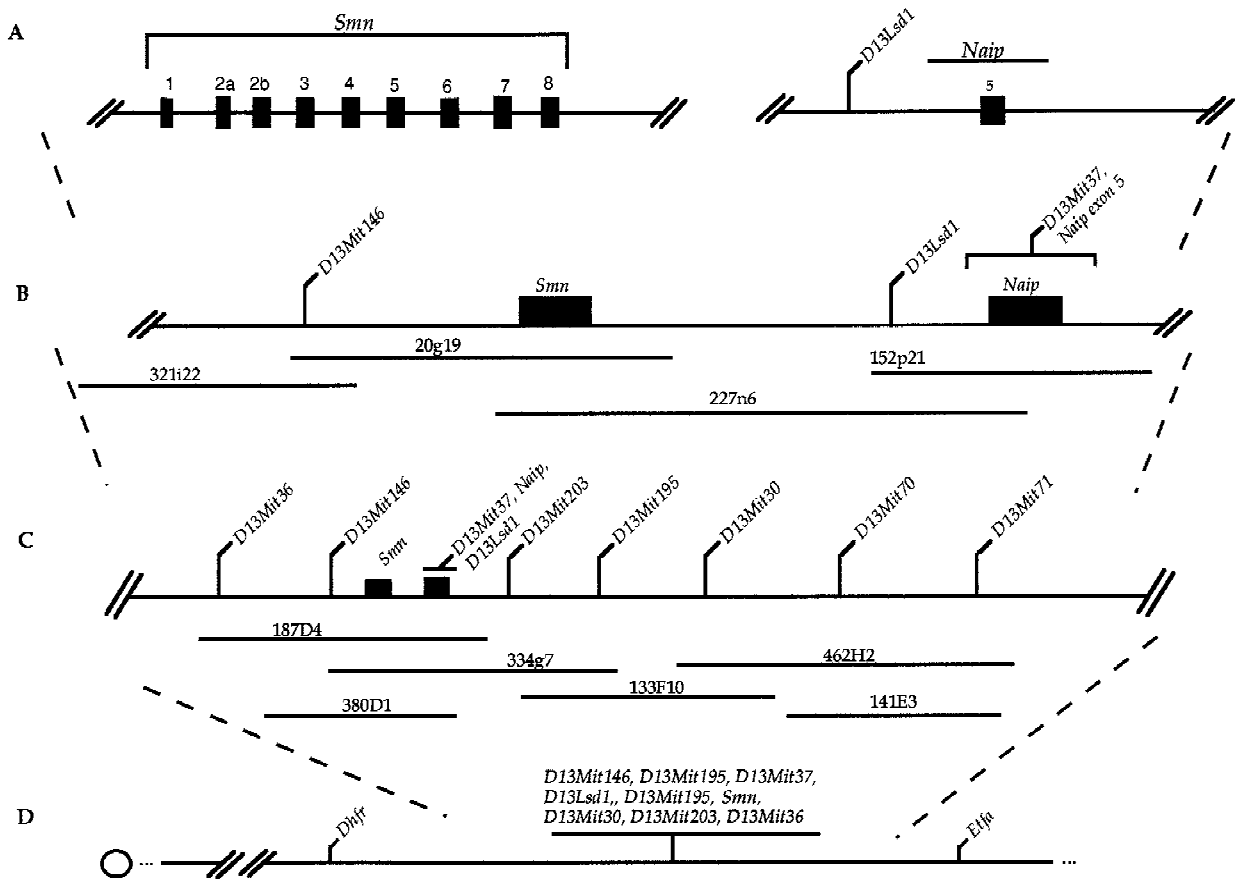

Figure 6 (*A*) A schematic diagram of the *Smn* gene and a portion of the *Naip* gene. The structure of the *Smn* and *Naip* genes was established by alignment to the homologous human genes. Note that *Naip* exon 5 and *D13Mit37* are represented multiple times in this area but are shown only once for simplicity. (*B*) BAC contig of the region containing the *Smn* and *Naip* genes. Shown is the minimal number of BAC clones needed to complete a contiguous array spanning the markers *D13Mit146–D13Mit37*. (*C*) Contiguous array of YAC clones spanning the region from *D13Mit36* to *D13Mit70*. Shown are only those YACs that were positive with two or more MIT markers mapping to this region. These YACs are negative for all other MIT markers, which suggests that they are not chimeric. The order of the markers from centromere to telomere was established by genetic and physical mapping. (*D*) Chromosome 13.

cDNA library (the kind gift of P. Gros, McGill University, Montréal, Québec, Canada) were plated, transferred to nylon membranes, and hybridized with a ^{32}P-labeled random-primed (Feinberg and Vogelstein 1983) human SMN exon 6–8 (518 bp) probe using standard methods (Sambrook et al. 1989). A λgt10 7.5-day whole embryo cDNA library (the kind gift of B. Hogan, Vanderbilt University Medical Center, Nashville, Tennessee) was screened by PCR using *Smn* exon 3 primers according to the method of Israel (1993). IMAGE Consortium (LLNL) cDNA clones 352779 and 419573 (Lennon et al. 1996) were identified by using the human SMN cDNA sequence as a query to search the National Center for Biotechnology Information (NCBI) EST database using the BLASTn program (Altschul et al. 1990). cDNA clones were sequenced on both strands using the double-stranded cycle sequencing system (GIBCO-BRL).

Isolation of Genomic BAC and YAC Clones

Using standard methods, human SMN exon 6–8 and human *Naip* exon 5 probes were used to screen high-density 129/SvJ BAC filters (Genome Systems). DNA pools of a 129/SvJ BAC library (Research Genetics) were screened by PCR using primers specific to *Smn* exon 2b (see Table 1). YAC clones were identified by searching the MIT database for MIT markers spanning the genetic map established by this work. DNAs from BAC and YAC clones were isolated as recommended by Genome Systems.

STS Content Mapping

All STS content mapping was performed by PCR. PCR amplification conditions for MIT markers were according to Dietrich et al. (1992). Novel STSs described in Table 2 were amplified using the conditions described below. YAC and BAC clones were analyzed for the presence or absence of STSs and SSLPs using single-colony picks or DNA obtained from liquid cultures of single colonies.

SSCA

SSCA was performed for each exon of the *Smn* gene using the

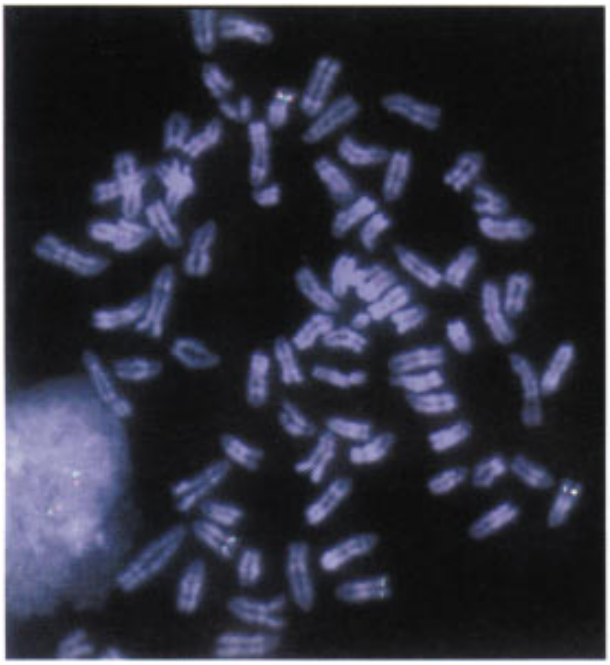

Figure 7 FISH mapping of BAC probes containing the *Smn* and *Naip* genes to mouse Chromosome 13. The BAC genetic marker *D13Mit300* was used as a hybridization probe to identify mouse Chromosome 13 accurately (D. Noya, X.-N. Chen, B. Birren, K. Devon, J.S. Lee, and J.R. Korenberg, unpubl.). The map assignment was corroborated by chromomycin A3 and distamycin A reverse banding. The differentially labeled *Smn* (digoxigenin; red signal) and *Naip* (biotinylated; green signal) probes were cohybridized onto metaphase chromosomes and unstimulated interphase cells prepared from strain 129. The FITC and rhodamine signals were mapped to the region MMU13D1-2.1. The MGD accession number for physical mapping of *Smn* and *Naip* is MGD-INEX-31. It should be noted that multiple copies of a region-specific repeat that contains at least *Naip* exon 5 was not resolved by FISH because of the size of the BAC used as probe for the *Naip* gene.

primers described in Table 1. PCR was performed in a total volume of 25 µl that contained 50 ng of genomic DNA or 2 ng of subcloned DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 µM each primer, 300 mM dATP, dGTP, and dTTP, 150 mM dCTP, 44 nM [^{32}P]dCTP, and 1 unit of *Taq* DNA polymerase (BRL). The final $MgCl_2$ concentration, annealing temperature, and product size for each exon is described in Table 1. Cycling conditions consisted of an initial 3-min denaturation step at 94°C, followed by 35 cycles of 94°C for 30 sec, annealing for 30 sec, and 72°C for 30 sec in a Perkin Elmer Cetus Thermocycler 1. Resultant PCR products were diluted fivefold with 0.1% SDS and 10 mM EDTA, mixed with an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% zylene blue), heated to 80°C for 5 min, snap-cooled on ice, and 5 µl was loaded onto a 0.5× MDE (AT Biochem)/0.6× TBE gel. To increase the probability of detecting single-base-pair substitutions, two different electrophoretic conditions were used: room temperature or 4°C at 7 W for 14–17 hr, depending on the size of each exon.

SSCA variants between SPRET/Ei and 129/SvJ, as well as C57BL/6JEi, were identified. Products from 50-µl PCRs were purified using Wizard Magic PCR preps (Promega) and sequenced directly using the double-strand cycle sequencing kit (GIBCO-BRL). These products were also cloned into a plasmid vector using a TA cloning kit (Invitrogen). Four independent clones from each sample were sequenced and the sequences compared to that obtained directly from the PCR product.

Genetic Mapping of *Smn*

Smn was genetically mapped by PCR analysis of DNA from 94 progeny of the Jackson Laboratory BSS interspecific backcross mapping panel (Rowe et al. 1994). Reaction and amplification conditions for *Smn* exon 2b primers (Table 1) were identical to those described above. The amplified products were diluted 1:1 with formamide loading buffer, heated at 80°C for 3 min, and 8 µl of each reaction was loaded onto a 6% denaturing polyacrylamide gel and electrophoresed at 2000 V for 3 hr. Gels were dried and exposed to Fuji film at −80°C overnight with two intensifying screens. *Smn* exon 2b amplified a product of 206 bp, 206 bp, and 199 bp using 129/SvJ, C57BL/6JEi, and SPRET/Ei genomic DNA, respectively.

Northern Blots

Northern blots (Clontech) containing ~2 µg of poly(A)$^+$ mRNA from mouse fetal and adult tissues were hybridized with a radioactive 1126-bp *Smn* cDNA (exons 2b–8) probe prepared by random priming (Feinberg and Vogelstein 1983). Hybridization was carried out in 7 ml of Rapid Hyb buffer (Clontech) at 65°C for 2 hr according to the manufacturer's instructions. The filters were washed sequentially at 65°C for 10–20 min in 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS; 0.5× SSC, 0.1% SDS; 0.2× SSC, 0.1% SDS, and 0.1× SSC, 0.1% SDS, respectively, and monitored with a Geiger counter between buffer changes. The filters were exposed to Fuji film at −80°C with two intensifying screens for varying lengths of time. After exposure, the filters were stripped and rehybridized with β-actin, washed, and exposed for 20 min.

RT–PCR

Tissues from a CD1/*Spf* female mouse were disrupted by a Polytron homogenizer in Trizol reagent (GIBCO-BRL), and total RNA was isolated according to the manufacturer's instructions. To analyze the *Smn* gene for alternatively spliced isoforms, 5 µg of total RNA from liver, kidney, brain, and spinal cord was reverse transcribed using an exon 8 Rev primer (see below) and 200 units of Superscript RT II (GIBCO-BRL) in a total volume of 25 µl according to the manufacturer's instructions. For each amplification, 3 µl of first-strand cDNA was used. *Smn* exons 1–4 or 1–7 were amplified using 2 pmoles of forward and reverse primers in a total volume of 25 µl and 1 unit of *Taq* polymerase (GIBCO-BRL). Amplification conditions were as follows: an initial 3-min denaturation step at 94°C, followed by 35 cycles of 94°C for 30 sec; 56°C for 30 sec; and 72°C for 40 sec in a Perkin Elmer Cetus Thermocycler 1. The resulting PCR products were electrophoresed in a 2% agarose/1× TBE gel. *Smn* exon 1–7 RT–PCR products were trans-

ferred to a Hybond N+ nylon membrane (Amersham), hybridized with a labeled *Smn* exon 1–2b probe and washed at high stringency in 0.1× SSC, 0.1% SDS, at 65°C for 45 min. The blot was exposed to Kodak X-OMAT film overnight at −80°C with two intensifying screens. Only the 860-bp PCR product corresponding to full-length *Smn* was identified. PCR primers (5′ → 3′) are as follows: exon 8 (reverse), gCACATTTgTgCTCAgTCACg; exon 1 (forward), ATggCgATGggCAgTggC; exon 4 (forward), AATgAAAgTCAAgTTTCCACA; exon 7 (reverse), gTATgTgAgCACTTTCCTTC.

FISH Mapping of *Smn* and *Naip* BAC Clones

DNAs from mouse BACs containing either the *Smn* (20g19) or *Naip* (152P21) genes were labeled with digoxigenin-11–dUTP and biotin-14–dATP, respectively, using a nick translation system (GIBCO-BRL). FISH analysis was performed essentially as described by Korenberg and Chen (1996). Briefly, in 10 µl of the hybridization mixture (50% formamide, 10% dextran sulfate; and 2× SSC), 100 ng each of biotinylated and digoxigenin-labeled DNA probes were mixed with 3 µg of human Cot 1 DNA, 4 µg of mouse Cot 1 DNA (GIBCO-BRL), and 3 µg of sonicated salmon sperm DNA to suppress cross-hybridization with repetitive sequences. The DNA probes were applied to denatured mouse chromosome slides prepared from female mouse spleen cells (strain 129) using a modification of the method described by Boyle et al. (1990) and Zhu et al. (1995). An independent control experiment was done that involved hybridization of the probes to mouse lymphocytes, where the majority of cells were assumed to be in G1 as the cultures were not treated with concanavalin A. Three washes were performed in buffer containing 2× SSC and 50% formamide for 5 min at 40°C, followed by three additional washes in 1× SSC for 5 min at 45°C. Hybridization signals were detected with avidin-conjugated fluoroscein isothiocyanate (Vector Labs) and sheep anti-digoxigenin–rhodamine. Amplification of FITC signals was achieved by using biotinylated–anti-avidin. Chromomycin A3 and distamycin A were used to counterstain individual chromosomes. The images were captured and stored using the Photometrics Cooled-CCD camera (CH250) and BDS image analysis software (ONCOR Imaging, Inc.).

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