GENOMICS **38,** 405–417 (1996)
ARTICLE NO. 0644

The Mouse Region Syntenic for Human Spinal Muscular Atrophy Lies within the *Lgn1* Critical Interval and Contains Multiple Copies of *Naip* Exon 5

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Received August 5, 1996; accepted October 17, 1996

Spinal muscular atrophy (SMA) is a relatively common, autosomal recessively inherited neurodegenerative disorder that maps to human chromosome 5q13. This region of the human genome has an intricate genomic structure that has complicated the evaluation of SMA candidate genes. We have chosen to study the mouse region syntenic for human SMA in the hope that the homologous mouse interval would contain the same genes as human 5q13 on a simpler genomic background. Here, we report the mapping of such a region to mouse chromosome 13 and to the critical interval for *Lgn1*, a mouse locus responsible for modulating the intracellular replication and pathogenicity of the bacterium *Legionella pneumophila*. We have generated a mouse YAC contig across the *Lgn1*/*Sma* interval and have mapped the two flanking gene markers for the human SMA locus, MAP1B and CCNB1, onto this contig. In addition, we have localized the two SMA candidate genes, SMN and NAIP, to the *Lgn1* critical region, making these two genes candidates for the *Lgn1* phenotype. Upon subcloning of the YAC contig into P1s and BACs, we have detected a large, low copy number repeat that contains at least one copy of *Naip* exon 5. Identification of the *Lgn1* gene will either provide a novel function for SMN or NAIP or reveal the existence of another, yet uncharacterized gene in the SMA critical region. Mutations in such a gene might help to explain some of the phenotypic variability among the human SMAs.

INTRODUCTION

The human spinal muscular atrophies (SMAs) are a group of clinically heterogeneous, autosomal recessive neurodegenerative disorders characterized by a progressive loss of spinal cord motor neurons and symmetrical, secondary skeletal muscle atrophy. Together, they constitute the second most common childhood neuromuscular disease in the Caucasian population with an incidence of approximately 1 in 10,000 live births (Pearn, 1980). The SMAs can be divided into four clinical forms, designated Types I–IV, which are defined by age of onset and disease severity. Patients with Type I SMA, also known as Werdnig–Hoffmann disease, fail to sit without assistance and often die before the age of 2 (Dubowitz, 1995). In stark contrast, adult-onset Type IV SMA patients present with mild proximal muscle weakness and often have a normal life expectancy (Rudnik-Schoneborn *et al.*, 1994).

All four SMA variants map to the same chromosomal location, 5q13, a region of the human genome that has proven to be unusually complex (Brahe *et al.*, 1995; Clermont *et al.*, 1995; Gilliam *et al.*, 1990; Melki *et al.*, 1990). Detailed analysis of this locus has revealed the presence of a 500-kb 5q-specific inverted duplication (Lefebvre *et al.*, 1995) as well as large, low copy number repeats with homology to sequences found elsewhere on chromosome 5 (Francis *et al.*, 1993; Thompson *et al.*, 1993). These repetitive elements make the SMA interval extremely unstable, as documented by the high frequency of *de novo* deletions in Type I patients (Melki *et al.*, 1994). In addition, the critical region contains several highly transcribed pseudogenes that have further confounded efforts to define relevant candidate genes (Selig *et al.*, 1995; Theodosiou *et al.*, 1994).

Two genes have been reported as SMA candidates based on deletion analysis in patients. The survival motor neuron gene (SMN) is a novel, ubiquitously expressed transcript that exists in two nearly identical copies on each human chromosome 5 (Lefebvre *et al.*, 1995). The telomeric copy of SMN is absent in 92–98% of Type I and II SMA patients and at least 82% of Type III patients (Cobben *et al.*, 1995; Hahnen *et al.*, 1995; Lefebvre *et al.*, 1995; Rodrigues *et al.*, 1995; Velasco *et al.*, 1996). However, 10 unaffected siblings and three

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0888-7543/96 $18.00

parents of SMA patients have recently been reported to have homozygous deletions in the telomeric SMN without clinical symptoms of the disease (Cobben *et al.*, 1995; Hahnen *et al.*, 1995; Wang *et al.*, 1996). These anomalies in genotype–phenotype correlation raise the question as to whether additional genes may be responsible for the SMA phenotype or may modulate the severity of the disease.

The neuronal apoptosis inhibitory protein gene (NAIP) is a strong candidate as a phenotypic modifier of SMA. This transcript is expressed in a variety of tissues and contains three baculovirus inhibition of apoptosis protein repeat (BIR) motifs common among a family of apoptosis inhibitory proteins (Roy *et al.*, 1995). NAIP has been shown to inhibit some forms of apoptosis *in vitro,* consistent with its putative role as a neuroprotective agent (Liston *et al.*, 1996). The gene is deleted in 43–68% of Type I SMA patients, with a lower percentage of deletions seen in less severely affected patients (Burlet *et al.*, 1996; Hahnen *et al.*, 1995; Roy *et al.*, 1995; Velasco *et al.*, 1996). While NAIP may play a role in modulating the severity of the disease, failure to identify a disease-causing mutation in all patients makes this transcript unlikely to be the sole SMA causative gene.

Refined physical mapping of the human SMA interval has been hindered by the instability and complexity of the 5q13 locus. A number of physical maps of the region have been reported, yet no consensus map that is consistent with all the data can be established (Lefebvre *et al.*, 1995; Roy *et al.*, 1995; Thompson *et al.*, 1993; Wang *et al.*, 1995). The positions of SMN and NAIP relative to one another also remain unclear. Furthermore, it is possible that an additional transcript may exist in the region containing these two genes. For these reasons, we have chosen to study the SMA syntenic region in the mouse, in the hope that such an interval might contain the same genes as human 5q13 without the unusual genomic structure.

We report here that the mouse homolog to the human spinal muscular atrophy locus maps to mouse chromosome 13 and contains *Lgn1,* a mouse locus involved in resistance to infection with *Legionella pneumophila* (Beckers *et al.*, 1995; Dietrich *et al.*, 1995). *L. pneumophila,* the causative agent of Legionnaires' disease and Pontiac fever, is a facultative intracellular parasite. Infection of macrophages by *Legionella* seems to be an important aspect of the pathogenesis of legionellosis (Ciancotto *et al.*, 1989). In particular, the ability to propagate *Legionella* intracellularly in primary macrophages and macrophage cell lines from a variety of mammalian sources has helped to advance the knowledge of the intracellular compartments inside of which the bacteria reside and replicate (Berger and Isberg, 1993; Horwitz, 1983, 1984, 1987).

While *Legionella* can grow in macrophages from many mammalian sources (Marra and Shuman, 1992), primary macrophages from most inbred mouse strains will not support the intracellular replication of *Legionella,* even though the bacteria gain entry to the cell (Yamamoto *et al.*, 1992). One exception to this is the strain A/J; macrophages from this inbred strain are permissive for bacterial replication (Yamamoto *et al.*, 1988). Crosses between A/J and a nonpermissive strain C57BL/6J demonstrated that permissiveness for bacterial replication segregates as a recessive single gene trait (Yamamoto *et al.*, 1991; Yoshida *et al.*, 1991). Subsequently, we and others mapped the locus that causes this trait, *Lgn1,* to mouse chromosome 13 (Beckers *et al.*, 1995; Dietrich *et al.*, 1995).

We have generated a detailed genetic and physical map of the *Lgn1*/*Sma* interval, with comparisons to the homologous region on human chromosome 5. We have mapped the mouse homologs of the genes flanking the human SMA critical region, *Ccnb1-rs13* (CCNB1) and *Mtap5* (MAP1B), as well as the homologs of the reported candidate genes within the human SMA interval, *Smn* and *Naip*. The mouse syntenic region is somewhat less complex than its human counterpart; nevertheless, there is evidence of duplications within the *Lgn1*/*Sma* interval that appear to have arisen independently in the mouse. Further analysis of the mouse interval should identify the gene responsible for *Legionella* susceptibility as well as any unidentified SMA candidate genes.

MATERIALS AND METHODS

Genetic mapping of Smn. A hybridization probe was prepared by PCR amplification of a human cDNA clone containing the entire SMN coding region (kindly provided by Dr. T. C. Gilliam, GenBank Accession No. U18423) and used to screen a mouse 129 genomic phage library (λFIX II, Stratagene, La Jolla, CA) under standard conditions (Sambrook *et al.*, 1989). Positive clones were purified, subcloned into Bluescript plasmid pBS II SK(+) (Stratagene), and sequenced using an Applied Biosystems automated sequencer. Primers were designed from this sequence using the program OLIGO (National Biosciences, Plymouth, MN) to amplify a 189-bp fragment from intron 1 of *Smn* (JMS 6, 5′-GACTGTAGAAGTAAGATAAACACTG-3′ and JMS 7, 5′-CAGTTGCCATACCATAAGACGAC-3′) (Gibco BRL, Grand Island, NY) using C57BL/6J and *Mus spretus* genomic DNA as templates in the presence of 1 μCi of [α-^{32}P]dCTP. An SSCP variant was detectable by resolving the PCR products on a 0.5× MDE gel (FMC, Rockland, ME) at 6 W for 12 h at room temperature. The primers were then tested on the 96-animal Jackson Lab (C57BL/6J × *M. spretus*) × *M. spretus* (BSS) backcross panel, and each animal was scored for the presence of the C57BL/6J allele (Rowe *et al.*, 1994). Genotyping data were sent to the Jackson Laboratories, where a map position was determined. In 7 animals, genotype was inferred from surrounding markers; in no case was any of these 7 animals recombinant with any surrounding markers on chromosome 13.

Genetic mapping of Lgn1. All A/J and C57BL/6J animals were obtained from Jackson Laboratories and bred at the Whitehead Institute for Biomedical Research. (A/J × C57BL/6J) × A/J backcross animals recombinant for the markers *D13Mit128* and *D13Mit70* were identified by genotype analysis of genomic DNA isolated from small tail biopsies (Dietrich *et al.*, 1992). The 21 animals identified in the screen were then bred back to A/J animals to isolate progeny that had inherited the identical recombinant chromosome. The parent recombinant animals and, frequently, one recombinant progeny-test animal from each line, were sacrificed by CO_2 asphyxiation. Their organs were harvested for DNA extraction, and bone marrow cells from the femur were isolated for *in vitro* differentiation into macrophages (Dietrich *et al.*, 1995). The cultured macrophages from each animal were tested for permissivity to intracellular replication of *L. pneumophila* as previously described (Dietrich *et al.*, 1995).

TABLE 1

STS Primers Used to Construct the *Lgn1* Physical Map

Locus name	Assay name	Primer sequences	C57BL/6J size
D13Mit36	A1126	ttgactctttaaaagactggtgtct cctggaagtgtatcctagtgg	140 bp
D13Die4	b7-1	ttgttgaaacctgtaggacatcc acttgggtccctgaaggtg	230 bp
D13Die21	b24-1	tttttaaattatcaaatagaagag tacagattgagtttggcccc	123 bp
D13Die8	b5-1	tttaaaaagaatattctgctcttc ccgtgatttttttcctttcgt	168 bp
D13Die20	b23-1	atatggtggtggctggaaaa tacacttgccatggcgc	147 bp
D13Die5	b13-2	cactccctcttacatgatgttcc cgaggataagcaactgtgagg	129 bp
D13Die15	18a12R	ggtacaacataaaaggaa agtgtttctcaagaagcaat	75 bp
D13Mit146	MT969	aagttacatttgtttttattttgtgtg gagcaaaacctaccagaagtgg	110 bp
D13Die9	CA7	gatctggcaggtncacgt aaacctgagctgtatcnctagc	157 bp
D13Die14	144c9R	aactaaacaggacacctctgca aaccaagaggctagacaaacag	72 bp
D13Die11	32b	acaatttggaggctggtgac tctggtcctcatgatggaat	64 bp
D13Die22	b15-1	ggacatggcaaacgctg ccatggcagctagttcacaa	138 bp
D13Die13	55	taactaagacacctccacagcc aggcctcagccactgcag	201 bp
D13Die6	b15-2	cacagtgcacaaaaccatcc aacttcaacaaggccacacc	124 bp
D13Die23	3-10-1	tgaagaaaagagggcaatgg gccaaacctcttgctctctg	314 bp
D13Mit37	B199	aactgtctccttcctgtttttcc agaccactgactttgcagtaagc	226 bp
	55-2	gatctctcccagcagtatttgg gctcaggaaaaaaaatggagg	240 bp
D13Die7	3-1-3	aatttctgtgtggttgtgtg ataaaccatccctagttgcctg	135 bp
D13Die1	32-1	gatcatcccacctncaggggc gatctgtgtgtgtctgtgtgcttgcatgag	140 bp
D13Die12	21b	ttgaaaaaaaaattgacccagt acattgacatccagcccaat	163 bp
D13Die3	59-1	acgacagcgtcttcgctaat gggcagcattgcagacat	219 bp
D13Die10	CA16	catgagttgccagtttatttacg tttttcgcaaaacctgtgttt	>500 bp
D13Mit203	MT2364	gggaacactgagacccaaaa agcacctctaaaggatcgagc	145 bp
D13Mit70	MPC1555	tctcaaaagcacttcttttctaca gagtgacgccgttggttatt	162 bp

Genotype analysis with additional microsatellite genetic markers was performed on DNA isolated from the organs of each animal using standard techniques (Ausubel *et al.*, 1989).

Construction of physical map. The STSs discussed in this paper are described in Table 1. Sublibraries were constructed from the YAC clones isolated in screens done at Research Genetics (Huntsville, AL): 144C9, 144A1, and 18A12. These small insert libraries were made by ligating *Mbo*I-digested yeast miniprep DNA into the *Bam*HI site of M13mp18. The clones were screened for the presence of (CA)*n* repeat loci by hybridization, and positive clones were sequenced according to the manufacturer's specifications using ABI fluorescent sequencing chemistry on 373 and 377 sequencing instruments. All the sequences were used to select STS primers using the program PRIMER (Whitehead Genome Center, Cambridge, MA). Those STSs that amplified mouse DNA were tested to see if they mapped back to the region by amplifying 20 ng of YAC miniprep DNA as previously described (Dietrich *et al.*, 1992). Microsatellite markers that successfully mapped back to the region were given the prefix "*D13Die*" and used to refine both the genetic and the physical map. The additional YAC clones were identified by PCR screening YAC library pools available at the Whitehead Genome Center (Haldi *et al.*, 1996; Kusumi *et al.*, 1993). The BAC clones were isolated from PCR pools for a mouse BAC library available at the Whitehead Genome Center (Shizuya *et al.*, 1992). The P1 clones were isolated by Genome Systems (St. Louis, MO) by PCR screening pools of the 129 BAC library (Pierce *et al.*, 1992).

SMA STS mapping. STSs from the mouse homologs of genes found in the human spinal muscular atrophy locus were generated as follows: *Mtap5* 5′ STS primers were designed to amplify a 179-bp fragment from exon 1 of *Mtap5* (GenBank Accession No. X51396) (F1, 5′-GGCGGGAGAGGAACACTTCT-3′ and R1, 5′-CTCGCCGACCACCACCAGCA-3′); *Mtap5* 3′ STS primers were designed to amplify a 204-bp fragment from exon 5 of *Mtap5* (F2, 5′-CTTTAGTCGGCAGTCTCC-

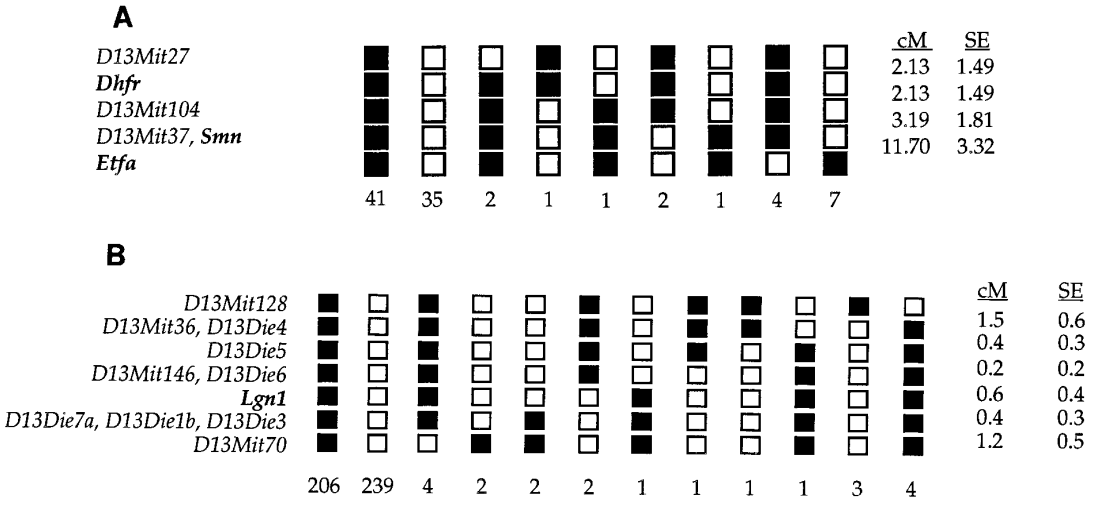

FIG. 1. Haplotype distribution frequency for *Smn*, *Lgn1*, and surrounding markers on mouse chromosome 13 derived from the backcross panels. Each column of boxes represents a chromosomal haplotype found in the backcross progeny. The number of progeny inheriting each chromosome is depicted below each column. cM, centimorgans; SE, standard error. (**A**) Mapping of *Smn* to a position recombinationally inseparable from *D13Mit37* on the 94-animal Jackson Laboratories (C57BL/6J × *M. spretus*) F_1 × *M. spretus* (BSS) backcross panel. Black squares represent the presence of a C57BL/6J allele, while white squares represent the presence of only the *M. spretus* allele. Seven typings from the *Smn* data and nine typings from the *Dhfr* data were inferred from nonrecombinant surrounding markers. Raw data for the cross with references for each mapping are available on the WWW at http://www.jax.org/resources/documents/cmdata. *Dhfr*, dihydrofolate reductase; *Etfa*, electron-transferring flavoprotein, alpha polypeptide; *Smn*, survival motor neuron. (**B**) Mapping of *Lgn1* between *D13Die6* and *D13Die3* on the 466-animal (A/J × C57BL/6J) × A/J backcross panel. Black squares represent the presence of a C57BL/6J allele, while white squares represent the presence of only the A/J allele. *Lgn1*, *Legionella pneumophila* susceptibility gene.

AGAT-3′ and R2, 5′-GGAAGCCTCTACCTGAGACACA-3′); *Ccnb1* primers were designed to amplify a 178-bp cDNA fragment or a 660-bp genomic fragment from the 5′ end of *Ccnb1* (GenBank Accession No. X58708) (F, 5′GCGGAGGAACGGCTGTTAGTT-3′ and R, 5′-ACTGTCACAGGCACACGCTTG-3′); *Smn* 5′ STS primers were designed to amplify a 189-bp fragment from intron 1 of *Smn* as described above; *Smn* 3′ STS primers were designed to amplify a 223-bp fragment from the 3′ UTR of *Smn* (F, 5′-TCAGCTCTGTCTCAGGAGATG-3′ and R, 5′-GTCACGACTGGGTAGACTGCC-3′); *Naip* exon 13 primers were designed to amplify a 405-bp fragment from exon 13 of the mouse *Naip* gene (*Naip* 15, 5′-CCGGTTTTTAGGTCCTCTGT-3′ and *Naip* 28, 5′-TTGCTTTCATGAGCAAAGTTT-3′); and *Naip* exon 5 primers were designed to amplify a 309-bp fragment from exon 5 of the human NAIP gene (GenBank Accession No. U19251) (JMS 13, 5′-AGCTCATGGATACCACAGGAGATG-3′ and JMS 14, 5′-ATATCCCTTGGACATAAAATGGC-3′). All STSs were typed on the YACs, BACs, and P1s by PCR amplification of 20 ng template, 50 ng each primer, 1× PCR buffer (Boehringer Mannheim, Indianapolis, IN), 200 μM dNTPs, and 2.5 U *Taq* DNA polymerase in a 25-μl reaction under the following cycling conditions: 3 min at 94°C, followed by 35 cycles of 30-s incubations at 55°C, 72°C, and 94°C, with a final 8-min extension at 72°C. PCR products were resolved on 2% agarose gels.

SSCP analysis of Naip exon 5. To detect individual alleles of *Naip* exon 5, SSCP analysis was performed using primers JMS 13 and JMS 14. Twenty nanograms of cloned DNA or 100 ng of genomic DNA was PCR amplified using 20 ng of each primer, 1× PCR buffer (Boehringer Mannheim), 200 μM dNTPs, and 0.5 U *Taq* DNA polymerase in a 10-μl reaction in the presence of 1–10 μCi [α-^{32}P]dCTP. Cycling conditions were the same as described above. Twenty-five microliters of stop solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added to each reaction prior to denaturation at 95°C for 5 min. Two to five microliters were loaded on a 0.5× MDE gel with 10% glycerol and electrophoresed in 0.6× TBE for 16–21 h at 6 W at room temperature. Gels were dried and autoradiographed.

Sequencing analysis of Naip exon 5 alleles. Naip exon 5 alleles were PCR amplified from either genomic DNA or P1 and BAC clones, subcloned into the TA cloning vector (Invitrogen, San Diego, CA), transformed into bacteria, and purified by standard protocols (Sambrook *et al.*, 1989). Individual clones were assayed by SSCP (as described above) to determine allele identity and sequenced on an ABI automated sequencer using *Taq* DNA polymerase cycle sequencing. Acquired data were analyzed using Sequencher software (Genecodes, Ann Arbor, MI) or Genetics Computer Group software (program manual for the GCG package, Version 8, September, 1994). Homology to known sequences was determined by comparison with GenBank on the National Center for Biotechnology Information BLAST Network Service (Altschul *et al.*, 1990).

RESULTS

Genetic Mapping of the Mouse Smn Gene to Mouse Chromosome 13

In an effort to identify the mouse region syntenic for human SMA, we isolated a genomic phage containing the mouse SMN homolog, *Smn*, from a mouse genomic library. An SSCP variant between C57BL/6J and *Mus spretus* from intron 1 of *Smn* enabled the mapping of *Smn* to a position recombinationally inseparable from *D13Mit37* in the Jackson Laboratory (C57BL/6J × *M. spretus*) F_1 × *M. spretus* (BSS) backcross (Fig. 1A) (Rowe *et al.*, 1994). This map location placed *Smn* in the vicinity of the *L. pneumophila* susceptibility locus, *Lgn1* (Beckers *et al.*, 1995; Dietrich *et al.*, 1995, 1996).

Refined Genetic Mapping of Lgn1

To define the position of *Lgn1* more precisely, flanking genetic markers *D13Mit128* and *D13Mit70* were used to identify 21 recombinant animals from a 466-animal (A/J × C57BL/6J) × A/J backcross population. *Legionella*-infected macrophages from these recombinant animals were assayed for permissiveness to bacterial replication.

TABLE 2

Phenotype of Animals Recombinant in the *D13Mit128–D13Mit70* Interval

Parent animal No.	Number of animals analyzed[a]	Bacterial CFU[b]	Phenotype[c]
150	4	18	Nonpermissive
171	1	8.4	Nonpermissive
176	2	3.9	Nonpermissive
204	3	47	Nonpermissive
261	3	1.8	Nonpermissive
295	2	41	Nonpermissive
422	3	26	Nonpermissive
549	3	25	Nonpermissive
678	1	1.8	Nonpermissive
763	1	21	Nonpermissive
211	4[d]	6600	Permissive
222	4	5000	Permissive
226	3	2500	Permissive
269	1	4700	Permissive
541	3	3200	Permissive
642	2	4200	Permissive
661	3	2100	Permissive
686	3	3100	Permissive
770	3	4400	Permissive
771	3	4300	Permissive
781	2	4500	Permissive

[a] Total includes the parent recombinant animal and any progeny isolated with the same recombinant chromosome from a backcross to A/J (see Materials and Methods).

[b] Total average bacterial CFU ($\times 10^3$) isolated from duplicate infected macrophage cultures of the parent animal after 6 days (see Materials and Methods). Unless otherwise noted, the progeny-test animals confirmed the phenotype assessment of the parent animal.

[c] Defined as an average of greater than 1.2×10^6 CFU isolated from the duplicate cultures.

[d] One of the progeny recombinant animals typed as resistant, with an average of 1.5×10^4 CFU at 6 days.

Macrophages from 11 of the recombinant animals were permissive for growth using a previously described assay (Dietrich *et al.*, 1995). Macrophages from 10 animals were nonpermissive, closely corresponding to the 1:1 phenotypic ratio that would be expected for a single gene recessive trait in a backcross (Table 2).

DNA from these recombinant animals was typed with additional polymorphic genetic markers from the region to refine the position of *Lgn1*. The haplotypes of the recombinant chromosomes, indicating the position of *Lgn1*, are shown in Fig. 1B. The order and relative distance of the previously reported markers (*D13Mit128, D13Mit36, D13Mit146, D13Mit70*) broadly agree with previous maps (Dietrich *et al.*, 1995, 1996; O'Brien *et al.*, 1995). The other markers shown in the map are microsatellite polymorphisms that were obtained by sequencing subclones of YACs in the *Lgn1* region. The closest flanking polymorphic genetic markers to *Lgn1, D13Mit146, D13Die6, D13Die7a, D13Die1b,* and *D13Die3,* are approximately 1 cM apart.

Construction of a Physical Map and Isolation of Additional Markers

Since *Lgn1* and *Smn* appeared to map close to one another, a physical map across the mouse *Lgn1* critical region was generated to determine whether it also contained the homologous genes from the human SMA locus. Markers linked to *Lgn1* and *Smn* (*D13Mit36, D13Mit146,* and *D13Mit37*) were used to isolate YACs for a chromosome walk. Small insert libraries generated from these YACs were then screened for additional polymorphic microsatellite markers that might further refine the *Lgn1* critical region. A total of 25 STSs were identified on 17 YACs spanning approximately 2 Mb (Fig. 2). Marker *D13Mit37,* which was tightly linked to *Smn* in the *M. spretus* backcross, mapped within the *Lgn1* critical region defined by *D13Die3* and *D13Die6.*

Defining a Physical Map of the Mouse Homolog for the Human SMA Locus

Since the *Lgn1* physical map construction revealed that a marker closely linked to the mouse *Sma* region (*D13Mit37*) was contained within the *Lgn1* YAC contig (Fig. 2), the same intron 1 primers used to map *Smn* genetically were utilized to PCR amplify *Smn* intron 1 (*Smn* 5′ STS) out of the *Lgn1* YACs (Fig. 3). This experiment confirmed the presence of *Smn* within the *Lgn1* interval. In addition, an STS derived from the 3′ UTR (exon 8) of *Smn* mapped to the same YACs as the 5′ STS from *Smn* intron 1 (Fig. 2).

To characterize the entire mouse *Sma* interval, the YACs were tested for the presence of the mouse homologs of genes known to reside in the human SMA region (Fig. 3). The flanking gene markers that define the genetic and physical map position of SMA in human are microtubule-associated protein 1B (MAP1B) and cyclin B1 (CCNB1) (Brzustowicz *et al.*, 1992; Lien *et al.*, 1991; Pines and Hunter, 1989; van der Steege *et al.*, 1995). STSs from the mouse MAP1B homolog, *Mtap5* (Noble *et al.*, 1989), mapped at the centromeric end of the contig, amplifying from YACs 144C9 and 361B12 (Figs. 2 and 3). The mouse CCNB1 homolog, *Ccnb1-rs13* (Lock *et al.*, 1992; Paterno and Downs, 1991), mapped to the telomeric end of the *Lgn1* contig, amplifying from YACs 386F2 and 334G7 (Figs. 2 and 3). Thus, the entire human SMA critical region is contained within the *Lgn1* contig, in an orientation opposite to that of the human with respect to the centromere.

Similar experiments amplifying an STS from the *Naip* gene gave more confusing results. Primers were designed from human NAIP exon 5 and used to PCR amplify *Naip* from mouse genomic DNA as well as from the *Lgn1* YACs. *Naip* exon 5 appeared to map to apparently nonoverlapping YACs within the *Lgn1* critical interval and distal to *Smn* (Figs. 2 and 3). An STS from the distal end of the *Naip* gene (exon 13) produced identical results (Fig. 2).

The Lgn1/Sma Region Contains a Direct Repeat

The human SMA locus is known to contain a large inverted repeat that can be detected by microsatellite markers displaying more than one allele per chromosome

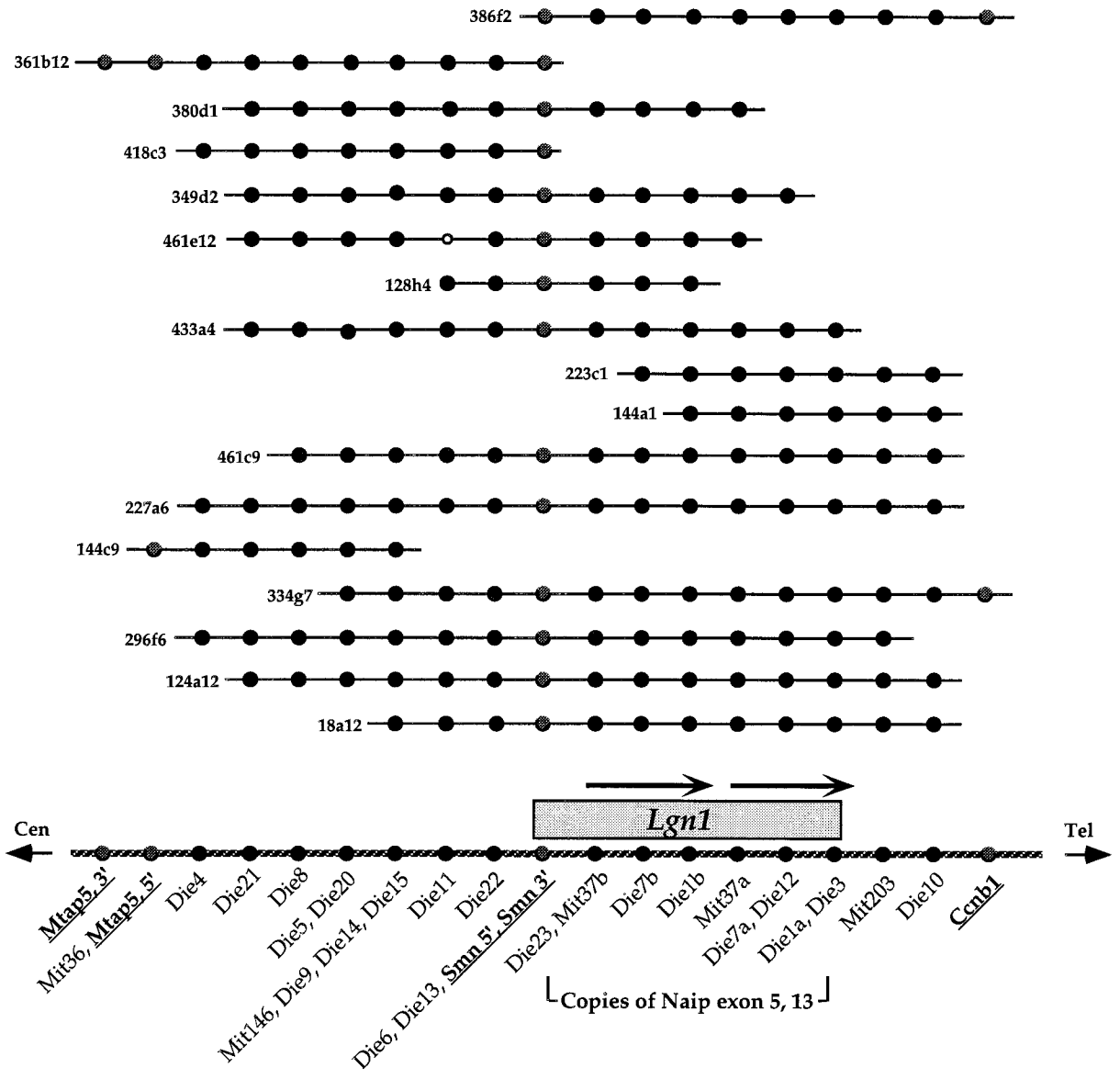

FIG. 2. YAC and STS contig of the *Lgn1*/*Sma* interval. YACs were ordered from centromere (left) to telomere (right) based on STS content as determined by PCR analysis. The names and origins of each of the YAC clones are discussed under Materials and Methods. STS primer information is shown in Table 1. Solid circles indicate STSs; shaded circles denote gene markers. The genes identified from the region are in boldface and underlined beneath the schematic of chromosome 13. The *Lgn1* critical region is depicted by the stippled rectangle bound by markers *D13Die6* and *D13Die3*. Although the distal border for *Lgn1* may extend as far proximal as *D13Die1b*, we have chosen to use the first nonrepetitive marker, *D13Die3*, as the *Lgn1* distal flank until the repeat region is completely characterized. The direct repeat of *D13Mit37–D13Die7–D13Die1* is indicated by the two arrows. Since *Naip* exon 5 and exon 13 map to multiple undefined loci within these repeats, their position on the contig is indicated in the bracketed region. There is only one discrepancy in the STS content of the map: marker *D13Die11* is not contained in YAC 461e12, as indicated by the open circle. We have placed this marker in a map position that minimizes the number of false positive and false negative results. However, the reverse order of cen–*D13Die22*–*D13Die11*–tel is just as likely as the order shown. With the exception of gene loci, all markers are abbreviated; the full name should include the prefix "*D13*." Cen, centromere; Tel, telomere.

(Burghes *et al.*, 1994; Melki *et al.*, 1994). Our inability to assign *Naip* a single position on the *Lgn1*/*Sma* contig prompted us to examine the mouse microsatellite data for similar evidence of large repeats in the mouse interval. Some YAC clones produced a smaller PCR product for *D13Mit37*, *D13Die7*, and *D13Die1* in addition to the predominant band (Fig. 4A; data not shown). These smaller products were also present in C57BL/6J genomic DNA. Both loci mapped to the *Lgn1*/*Sma* contig, forming a direct repeat of *D13Mit37–D13Die7–D13Die1* (Fig. 2). Since three bands were amplified from 129/SvJ DNA with *D13Die7*, there may be more than two copies of *D13Die7* in that strain, raising the possibility that the local repeat structure may vary between individual inbred strains.

Lgn1/Sma Map in BAC and P1 Clones

To obtain a higher resolution physical map and to clone the *Lgn1*/*Sma* interval into vectors more amena-

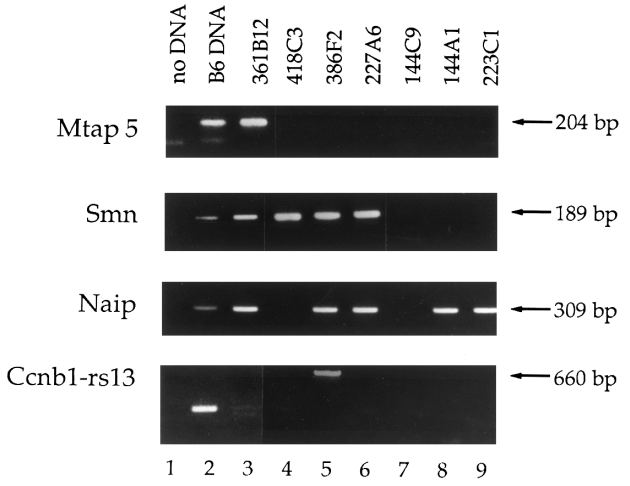

FIG. 3. Genes from the human SMA interval are present in the *Lgn1* YAC contig. PCR primers were designed to amplify fragments from the mouse homologs of the human genes flanking or contained within the 5q13 SMA interval (Materials and Methods). YACs from the *Lgn1* contig were assayed by PCR analysis for the presence of each one of these genes. The expected band for each gene marker is indicated at the right with an arrow and its appropriate size. Each band was excised from a 2% low melt agarose gel, purified, and sequenced to confirm the identity of the fragment. Lane **1,** no DNA template; lane **2,** 100 ng C57BL/6J (B6) genomic DNA; lanes **3**–**9,** 20 ng of C57BL/6J-derived YAC DNA. In the case of *Ccnb1-rs13*, a different size band was amplified in the genomic lane than in the YACs. The 300-bp genomic band has no sequence identity to *Ccnb1*, while the 660-bp YAC band can also be seen very faintly in genomic DNA and contains the predicted 180 bp of *Ccnb1* cDNA sequence plus 480 bp of intronic sequence flanked by consensus 5′ and 3′ splice sites (Paterno and Downs, 1991). The position of each of the YACs and the gene content of the remaining YACs in the contig are shown in Fig. 2.

ble to manipulation than YACs, BAC and P1 clones were isolated by screening libraries for local STSs from the region. The resulting map is generally consistent with the YAC mapping data (Fig. 5). Two alleles of *D13Die7* were present in the contig, each mapping to separate P1 clones. Markers spanning the repeat region were unable to be ordered relative to one another due to the highly homologous nature of the repeat units (see below).

Both 5′ and 3′ *Smn* STSs mapped to the same subset of P1s as *D13Die6*, the proximal flanking marker for *Lgn1* (Fig. 5). Therefore, the entire *Smn* gene lies either adjacent to or within the *Lgn1* critical region. When *Naip* exon 5 and exon 13 were positioned on the P1/BAC contig, these exons were once again present on apparently nonoverlapping clones, suggesting that they might be present in multiple copies.

The Lgn1/Sma Interval Contains Multiple Copies of Naip

In an attempt to enumerate the copies of *Naip* exon 5, PCR products were examined by SSCP analysis (Fig. 4B). SSCP analysis of C57BL/6J genomic DNA produced a minimum of eight bands, implying that *Naip* exon 5 is present in at least four copies in the mouse genome (Fig. 4B, lane 2). Since each band in genomic DNA is also present in *Lgn1* YAC 227A6 (Fig. 4B, lane 3), all of the *Naip* copies appear to have arisen from the *Lgn1*/*Sma* interval and not from another mouse locus.

When SSCP analysis was performed on the P1 and BAC clones from the contig, individual *Naip* exon 5 alleles segregated with different P1 clones (Fig. 4B, lanes 4–11). BAC 13021 appears to have three copies of *Naip* exon 5 (Fig. 4B, lane 5, corresponding to alleles 1, 3, and 6) and overlaps with P1s 8378 and 7745 (Fig. 4B, lanes 6 and 7, alleles 6 and 3, respectively). P1s 7744 and 9045 (Fig. 4B, lanes 8 and 9, alleles 2 and 4, respectively) were each found to have an additional, distinct copy of *Naip* exon 5. The presence of different *Naip* exon 5 alleles on P1 clones 7745, 7744, and 9045, which otherwise appear to be nearly identical in STS content, implies that these P1s are nonoverlapping or partially overlapping copies of a repeat unit containing at least *Naip* exon 5 and 13, *D13Mit37*, *D13Die7*, and *D13Die1* (Fig. 5).

Two additional bands appeared to be unaccounted for in genomic DNA (Fig. 4B, lane 4). To identify this remaining allele, a *Naip* exon 5 PCR product was subcloned from 129 genomic DNA and examined by SSCP analysis for the presence of novel alleles. Alleles presumably created by mutation during PCR were present in only 1 of 24 analyzed subclones, while previously identified alleles were found in up to 4 of 24 sampled clones. One new allele (Fig. 4B, lane 10) was also found in 4 subclones, and its SSCP migration pattern was consistent with that of the remaining genomic allele (Fig. 4B, lane 4). These data suggest that *Naip* exon 5 exists in at least six copies in the mouse genome. This contrasts with human NAIP exon 5, which has been reported to be unique and produces only a single pair of bands on SSCP analysis (Fig. 4B, lane 1). Since this analysis was performed on only one exon of the mouse *Naip* gene, these copies have been designated *Naip*-related sequences 1 through 6 (*Naip-rs1–6*), until it can be determined whether any of these genes produce a functional transcript.

Sequence Evolution of Naip Exon 5

Each unique *Naip* exon 5 allele was subcloned from the BAC and P1 clones and sequenced for comparison (Fig. 6A). The six alleles show 76% identity to human NAIP exon 5 at the nucleotide level and 79% homology (67% identity) at the protein level. Within the mouse, the alleles appear to fall into two distinct classes (Fig. 6B). *Naip-rs1* and *6* are 98% identical to one another, but only 94% identical to the other four alleles. Similarly, *Naip-rs2*,*3*, and *4* have 99% identity, differing from one another by only 1 bp. *Naip-rs5* is most closely related to *Naip-rs2–4*, with 97% homology to these three alleles. Interestingly, all six copies of *Naip* exon 5 could hypothetically encode parts of functional proteins, since none of the observed polymorphisms introduce stop codons. In addition, none of the allelic changes alter amino acids known to be conserved in

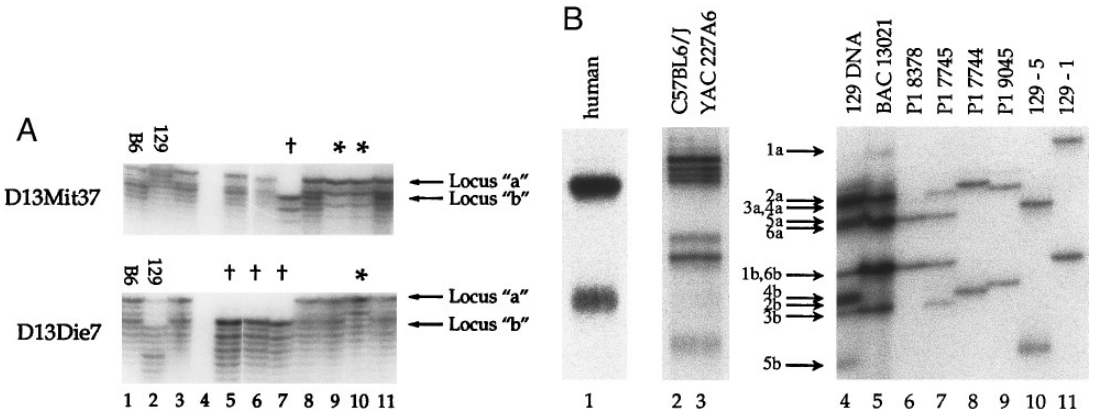

FIG. 4. (A) Duplications of *D13Mit37* and *D13Die7* in the *Lgn1*/*Sma* interval. The indicated microsatellite markers were typed on mouse genomic DNAs from C57BL/6J (B6) and 129/SvJ (129), and on the YAC clones in the *Lgn1*/*Sma* contig. The order of the YAC clones on the gel from left to right is 386F2, 361B12, 380D1, 461E12, 128H4, 433A4, 223C1, 144A1, and 461C9. For both markers, B6 DNA yields two prominent bands, "a" and "b". The lanes marked with an asterisk (*) contain YACs that have only locus a, whereas the lanes marked with a dagger (†) contain YACs that have only locus b. When these five YACs (lanes **5, 6, 7, 9, 10**) are placed in context with each other (Fig. 2), the two markers appear to form a direct repeat *D13Mit37b–D13Die7b–D13Mit37a–D13Die7a*. Three bands appear to be present in the 129 lane for *D13Die7*, raising the possibility that there are at least three alleles of this marker in strain 129/SvJ. **(B)** SSCP analysis of *Naip* exon 5. Primers were designed to amplify a 309-bp fragment from human NAIP exon 5 and used for SSCP analysis to detect multiple copies of this exon in human and mouse DNA. Lane **1,** human genomic DNA amplifying a single copy of NAIP exon 5. Lane **2,** C57BL/6J DNA; lane **3,** C57BL/6J-derived YAC 227A6 DNA spanning the *Lgn1*/*Sma* interval. A minimum of 8 bands (four alleles) are present in strain C57BL/6J. Lanes **4–9,** *Naip* exon 5 alleles in strain 129/SvJ (129) and P1 or BAC clones derived from strain 129. Arrows at the left of the panel point to individual bands; corresponding numbers refer to individual alleles. Alleles are numbered 1–6 starting from the top of the gel with "a" representing the upper band of the allele and "b" the lower band. A minimum of 12 bands (six alleles) are present in 129 genomic DNA; each allele can be separated from the other alleles in the corresponding P1 lanes. For example, allele 6 (bands 6a, 6b) segregates to P1 8378 (lane **6**). BAC 13021 (lane **5**) contains alleles 1, 3, and 6; P1 7745 (lane **7**) contains alleles 3 and 6; 7744 (lane **8**) has allele 2, and 9045 (lane **9**) has allele 4. Lanes **10** and **11,** individual subcloned *Naip* exon 5 alleles. Subcloned allele 5 (lane **10**) is present in 129 DNA (lane **4**) but not in any of the P1 or BAC clones. Subcloned allele 1 (lane **11**) is present in 129 DNA and BAC 13021 (lanes **4** and **5**). Upon overexposure of the film, allele 1 can also be visualized in P1 7745.

the putative first BIR domain of NAIP (Liston *et al.*, 1996).

DISCUSSION

Comparative genomic analysis is an extremely useful tool in molecular biology. The most common form of genomic comparison is that of DNA and protein sequence database searches, which often reveal structural and functional homologies between genes isolated from different organisms. The opportunity for and scope of genomic comparisons will increase as the genomes of several organisms are mapped and sequenced.

We have obtained data that allow the comparison of which specific homologous genomic regions in mouse and human contain genes important for seemingly different inherited phenotypes: spinal muscular atrophy in human and susceptibility to infection by *L. pneumophila* in mice. While the pathophysiological relationship between these two phenotypes, if any, is unknown, a comparative map of the syntenic regions should facilitate the identification of features important for understanding the molecular genetic basis of both traits.

The mouse distal chromosome 13 and human 5q11–q13 regions have some fundamental similarities. The mouse *Lgn1*/*Sma* region retains the same gross gene organization as its human counterpart, despite an inversion relative to the centromere (Fig. 7). *Mtap5*, like the human MAP1B, is oriented 5′ to 3′ away from SMA and represents the centromeric flank for the *Sma* region in the mouse (Wirth *et al.*, 1993). *Ccnb1-rs13*, whose human counterpart CCNB1 maps just proximal to the SMA critical interval (van der Steege *et al.*, 1995), lies in the distal part of the contig and represents the chromosome 13 region telomeric flank. The two human SMA candidate genes, SMN and NAIP, map within the center of the *Lgn1* contig and sublocalize to common BACs and P1s.

Interestingly, while *Smn* and *Naip* are both present in the human and mouse regions, their organization appears to be different in each species. SMN is duplicated in human, while mouse *Smn* appears to exist as a unique locus. However, since the human SMN copies are nearly identical to one another, further analysis must be performed to examine whether there is a second mouse *Smn* locus on chromosome 13. As for NAIP, exon 5 is thought to be present only in the functional telomeric copy in human (Roy *et al.*, 1995). In contrast, we have found that mouse *Naip* exon 5 is repeated multiple times within the *Lgn1*/*Sma* region. Although we cannot determine whether the repeats represent functional copies of *Naip*, alternatively spliced exons, or nontranscribed pseudogenes, we believe that there is at least one full-length *Naip* gene in the contig, based on the finding of *Naip* exon 13 in the *Lgn1*/*Sma* interval.

The most obvious difference between the mouse and the human regions is the repeat structure; the mouse

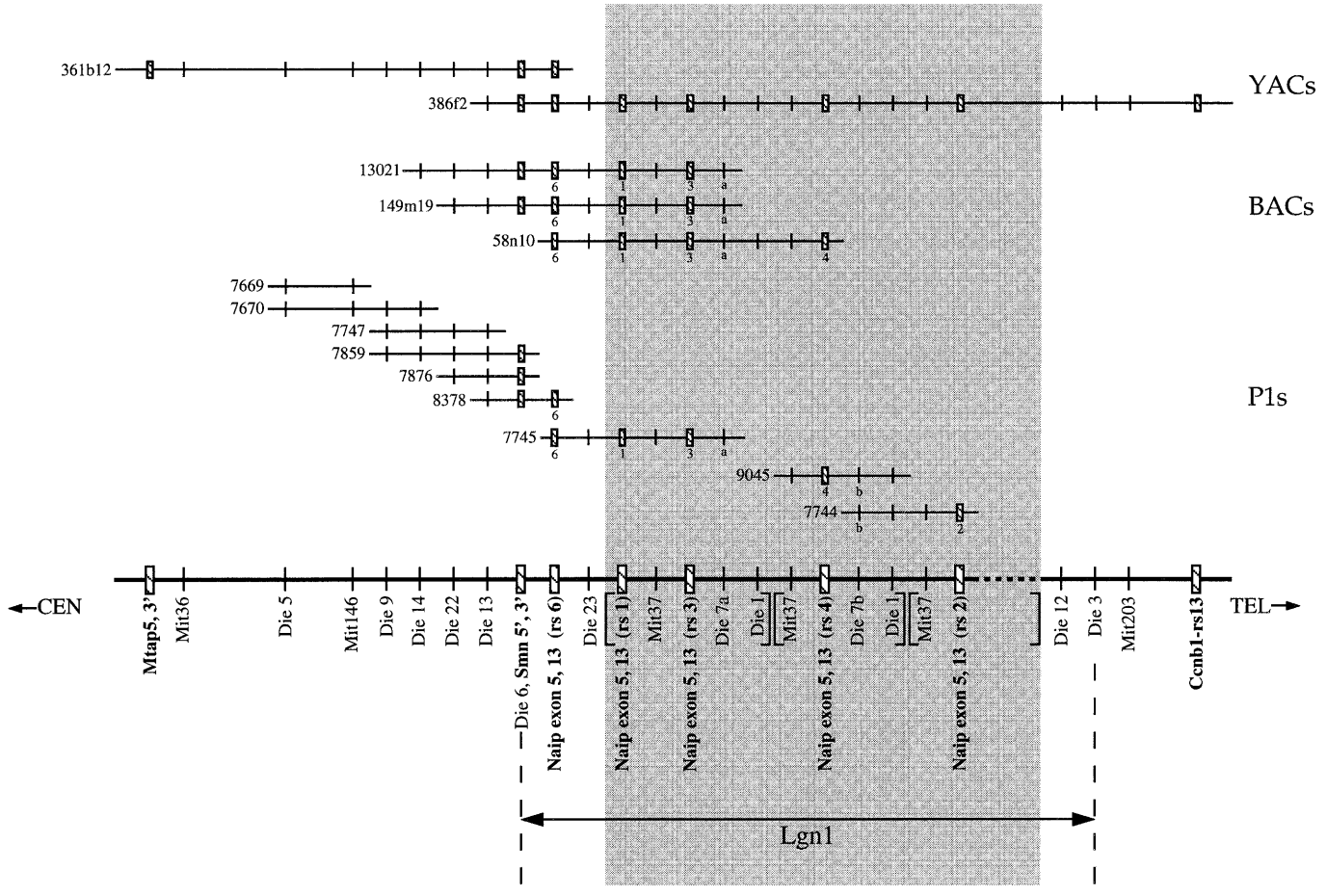

FIG. 5. BAC and P1 map of the *Lgn1/Sma* interval. BACs and P1s were analyzed by PCR for the presence of the STSs in Table 1. Two YACs spanning the *Lgn1/Sma* interval are included for reference. Vertical lines indicate STSs; boxes denote gene markers. The order of some STSs was presumed based on their order in the YAC map. Numbers underneath the boxes for *Naip* exon 5 indicate the allele contained within each clone as defined in Fig. 4B. The dark line at the bottom of the figure depicts mouse chromosome 13 in strain 129. The extent of the *Lgn1* critical region is indicated by the arrows and vertical dotted lines. The gray shaded area denotes the approximate borders of the repeat region. There is a gap in this region, since *Naip-rs5* is not present on the map. P1s 9045 and 7744 are depicted as partially overlapping clones, although formally they may lie on distinct, nonoverlapping repeat units. The two 129 alleles of *D13Die7* indicated as "a" and "b" refer to Fig. 4A, but do not necessarily correlate with the C57BL/6J alleles in Fig. 2. Markers indicated in brackets cannot be definitively ordered relative to one another due to the highly homologous nature of the repeats. CEN, centromere; TEL, telomere.

has a direct repeat, whereas the human repeats are inverted. This finding, in addition to differences in SMN and NAIP copy number between the two species, suggests that the human and mouse repeats arose as independent amplification events in closely linked, but distinct loci after the divergence of mouse from human. Since both SMA intervals appear to have undergone simultaneous gene expansion, there may be an unstable element common to the two regions that renders them prone to duplication. Identification of the junction between repetitive and unique sequences within the mouse *Lgn1/Sma* interval may reveal such an element at the repeat border, as has been suggested from sequence analysis of other multigene family loci (Pavelitz *et al.*, 1995).

Maintenance of repeat homology within multigene families is known to occur by two mechanisms: gene conversion and unequal crossing over. The former takes place either in tandem arrays or in gene clusters, where repeats are spaced by nonconserved sequences, and involves a unidirectional replacement of one allele for another via DNA recombination and repair. The latter is reserved for tandemly arrayed repeats and generates variations in repeat number each time a recombination event occurs (Graham, 1995). The human repeats show marked heterogeneity in the normal population; this instability has been proposed as a mechanism for generating disease-causing deletions of SMA genes (Melki *et al.*, 1994; Theodosiou *et al.*, 1994). Preliminary evidence suggests that there may be similar heterogeneity in mouse repeat copy number between different inbred strains. This observation supports the role of unequal crossing over in the maintenance of repeat homology in the *Lgn1/Sma* region (Figs. 4A and 4B). If there is such variation, it should be possible to detect and define the differences through the study of inbred strains of mice. Such studies may provide insight into the mechanism of repeat variability that might account for both the *Lgn1* phenotype and the variable severity of human SMA.

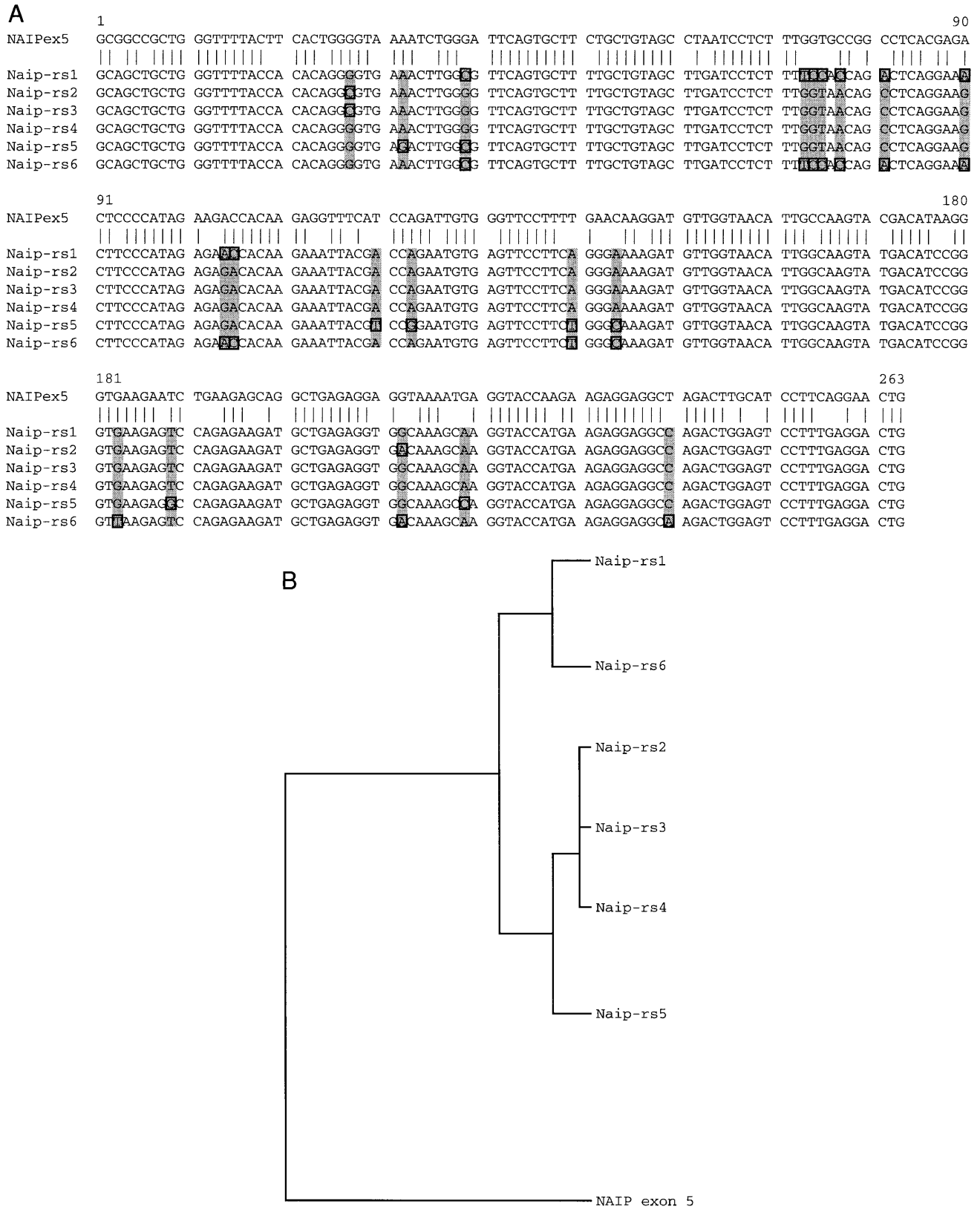

FIG. 6. Sequence comparison of *Naip* exon 5-related sequences (*Naip-rs1–6*). (**A**) Individual *Naip* exon 5 alleles were subcloned out of P1, BAC, or 129 genomic DNA and sequenced (Accession Nos. U66324–U66329). The allele numbers for the six mouse sequences correspond to alleles 1–6 identified in Fig. 4B. Vertical lines indicate nucleotide homology between the mouse sequences and human NAIP exon 5 (NAIPex5) sequence (Roy *et al.*, 1995). Shaded lines highlight polymorphisms among the mouse alleles, and nonconsensus bases are boxed. (**B**) Dendrogram of mouse *Naip* exon 5 alleles and human NAIP exon 5. Levels of homology were determined using the program PILEUP in the GCG software package.

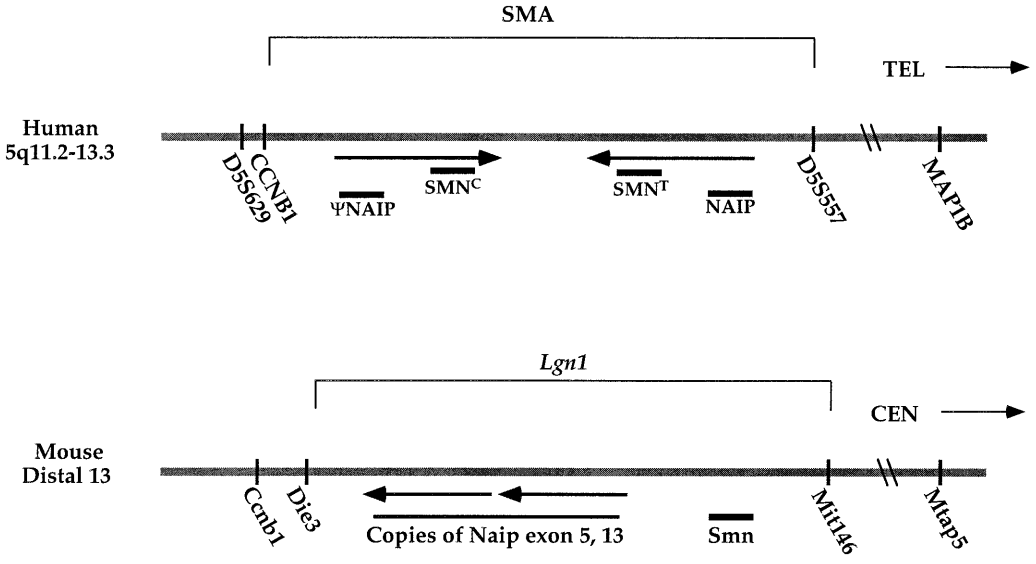

FIG. 7. Comparison of human and mouse *Lgn1*/*Sma* regions. Human chromosome 5q is depicted by the top line (Lefebvre *et al.*, 1995; Roy *et al.*, 1995) and mouse chromosome 13 by the bottom line. Homologous flanking gene markers and species-specific flanking microsatellite markers are indicated along each chromosome. Large brackets depict the critical regions for the SMA interval in human and the *Lgn1* interval in mouse. Arrows represent the repeat structure in each species. The positions of SMA candidate genes SMN and NAIP and their mouse homologs are indicated by black hashes. SMN^C, centromeric SMN; SMN^T, telomeric SMN; ΨNAIP, proposed NAIP pseudogene. Distances are not to scale.

While SSCP analysis suggests that there are at least six copies of *Naip* exon 5 in the repeat region, other repetitive markers appear to have only two or three alleles (Figs. 4A and 4B). This discrepancy could be due to the highly homologous nature of the repeats, as some repeats may have identical copies of these markers. Alternatively, there might be multiple copies of *Naip* exon 5 per repeat, since the six *Naip*-related sequences appear to fall into two classes (Fig. 6), possibly analogous to the two alleles detected by markers *D13Mit37*, *D13Die7*, and *D13Die1*. Isolation of additional BACs spanning more than one repeat unit will allow ordering of the repeats and should provide the framework upon which to understand the spatial relationship among the different *Naip*-related sequences.

We have narrowed the *Lgn1* critical region to a genetic distance of 1 cM on mouse chromosome 13 and have generated a physical map spanning the interval. Interestingly, the physical distance across the *Lgn1* critical region may be much smaller than what would be predicted by the genetic distance; *Lgn1* flanking markers from both sides fall on a single 140-kb BAC (Fig. 2). It is possible that the large, highly homologous repeats within the interval could increase the local recombination frequency. However, since the distal flanking markers for *Lgn1*, *D13Die1* and *D13Die7*, lie within the repeat elements (Figs. 2 and 4A), the marker loci detected in BACs 13021 and 149M19 may not represent the true distal end of the critical region. For this reason, we have chosen *D13Die3* as the definitive distal flanking marker for *Lgn1*.

Based on their positions within the *Lgn1* critical region, the mouse SMN and NAIP homologs are strong candidates for *Lgn1*. Both genes are expressed in a variety of tissues and therefore may have functions other than their putative role in motor neuron survival. Since the biological functions of SMN and NAIP are still unclear, discovery of another phenotype associated with either gene would considerably enhance the present understanding of that protein's normal cellular function. If one of these two genes is not responsible for the *Lgn1* phenotype, then there must be at least one additional gene in the *Lgn1*/*Sma* interval. Such a gene might not only explain *Legionella* susceptibility, but also could be the remaining SMA phenotype-modifying gene that would account for the clinical variability of the human SMAs. In either case, the continued positional cloning of *Lgn1* should provide important insight into the pathogenesis of spinal muscular atrophy.

ACKNOWLEDGMENTS

The authors thank Mary Ann Haldi for providing YAC clones, Bruce Birren and Keri Devon for help with screening the mouse BAC library, Jim Hudson at Research Genetics for excellent service for oligonucleotide primers and YACs, Zeeshan Husain for assistance with YAC screens, and Genome Systems for providing P1 clones. We recognize Dick Bennett and Gigi Bang as well as the HHMI sequencing facility for expert assistance with sequencing and thank Lucy Rowe and Mary Barter from the Jackson Laboratories for the BSS mapping panel and for their assistance in data analysis. We are grateful to Eric Lander, Elizabeth McNally, and Carsten Bonnemann for their insightful discussions throughout the progress of this work. Many thanks to Helene Sadoulet-Puccio, Emanuela Gussoni, Kristine Jackson, Brent Stockwell, and Marta Taylor for critical review of the manuscript. J.M.S. is a Howard Hughes Medical Institute Predoctoral Fellow. L.M.K. is supported by NINDS Grant NS 23740. L.M.K. and W.F.D. are both Investigators of the Howard Hughes Medical Institute.

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