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ARTICLE

The human centromeric survival motor neuron gene (*SMN2*) rescues embryonic lethality in *Smn*$^{-/-}$ mice and results in a mouse with spinal muscular atrophy

Umrao R. Monani[1], Michael Sendtner[5,+], Daniel D. Coovert[1,+], D. William Parsons[2], Catia Andreassi[1], Thanh T. Le[1], Sibylle Jablonka[5], Berthold Schrank[5], Wilfred Rossol[5], Thomas W. Prior[2], Glenn E. Morris[6] and Arthur H.M. Burghes[1,3,4,§]

Departments of [1]Neurology, [2]Pathology and [3]Medical Biochemistry, College of Medicine and [4]Department of Molecular Genetics, College of Biological Sciences, Ohio State University, Columbus, OH 43210, USA, [5]Department of Neurology, University of Würzburg, Josef-Schneider Strasse 11, D-97080 Würzburg, Germany and [6]MRIC, Biochemistry Group, Northeast Wales Institute, Wrexham, UK

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Proximal spinal muscular atrophy (SMA) is a common motor neuron disease in humans and in its most severe form causes death by the age of 2 years. It is caused by defects in the telomeric survival motor neuron gene (*SMN1*), but patients retain at least one copy of a highly homologous gene, centromeric *SMN* (*SMN2*). Mice possess only one survival motor neuron gene (*Smn*) whose loss is embryonic lethal. Therefore, to obtain a mouse model of SMA we created transgenic mice that express human *SMN2* and mated these onto the null *Smn*$^{-/-}$ background. We show that *Smn*$^{-/-}$;*SMN2* mice carrying one or two copies of the transgene have normal numbers of motor neurons at birth, but vastly reduced numbers by postnatal day 5, and subsequently die. This closely resembles a severe type I SMA phenotype in humans and is the first report of an animal model of the disease. Eight copies of the transgene rescues this phenotype in the mice indicating that phenotypic severity can be modulated by *SMN2* copy number. These results show that SMA is caused by insufficient SMN production by the *SMN2* gene and that increased expression of the *SMN2* gene may provide a strategy for treating SMA patients.

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is the most common inherited cause of childhood mortality with an incidence of 1 in 10 000 live births (1,2). It is an autosomal recessive disorder that is characterized by the destruction of the α motor neurons in the spinal cord. Based on the clinical severity and age at onset, the childhood SMAs have been divided into three types (3,4). Type I SMA is the most severe form with onset before 6 months and death occurring by the age of 2 years. Type II SMA is intermediate in severity with onset before 18 months of age and patients never gaining the ability to walk. Type III SMA is the mildest form of the disease with onset after 18 months. Type III SMA patients are able to walk. SMA is caused by mutations in the telomeric survival motor neuron gene (*SMN1*) but not the centromeric survival motor neuron gene (*SMN2*) (5–9). However, the *SMN* transcript is encoded by both genes and SMA patients do produce low levels of SMN protein from the *SMN2* gene (5,10,11). Species other than man have only one *SMN* gene, which is the equivalent of the human *SMN1* gene (12,13). In mice, a homozygous knockout of the *Smn* gene results in early embryonic lethality following massive cell death (14). This, coupled with the fact that SMA patients lacking *SMN2* have never been reported, suggests that SMN plays an essential role during embryonic development.

We and others (15,16) have previously shown that sequence differences in the promoters of the *SMN* genes do not explain the difference in function between them. Instead the critical difference between the two genes is a single nucleotide change in exon 7 that causes this exon to be spliced out of the majority of the transcript from *SMN2*. On the other hand, most of the transcript from *SMN1* is full length (17,18). Patients with milder forms of SMA have more copies of the *SMN2* gene than those with more severe forms of SMA, indicating that it can act as a phenotypic modifier (19, and references therein).

[+]These authors contributed equally to this work

[§]To whom correspondence should be addressed at: Department of Medical Biochemistry, 363 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, USA. Tel: +1 614 688 4710; Fax: +1 614 292 4118; Email: burghes.1@osu.edu

The 38 kDa SMN protein is ubiquitously expressed and is found in both cytoplasm and nucleus. Intranuclear SMN is found concentrated in aggregates called gems (20). The exact function of SMN remains unknown, but it has been shown to interact with the protein SIP-1 and several spliceosomal snRNP core proteins (21,22) and to play a critical role in spliceosomal snRNP assembly in the cytoplasm, most likely functioning in the regeneration/recycling of snRNPs and other splicing factors (23). In type I SMA patients SMN protein levels are severely reduced and cells lack gems (10,11).

Although SMN is ubiquitously expressed, the exact reasons why motor neurons are selectively affected are not clear. To understand further the molecular pathology of SMA it is important to understand the role of exon 7 in SMN, the effects of SMN dosage on motor neuron survival and whether *SMN2* is capable of rescuing early embryonic lethality in the mouse $Smn^{-/-}$ knockout. This can be addressed by making a mouse model of SMA. To generate such a model, we have introduced the entire human *SMN2* gene onto the null $Smn^{-/-}$ background. This mimics the situation in human SMA patients where SMN is present at all times albeit at greatly reduced levels. We show that when the *SMN2* transgene is present in low copy number on the $Smn^{-/-}$ background, a mouse with pathology similar to type I SMA patients results. This is the first report of an animal model of human SMA.

RESULTS

Construction of transgenes and genotype–phenotype analysis. We have previously shown that the entire human *SMN2* gene including its promoter lies on a 35.5 kb *Bam*HI fragment in the genomic clone PAC 215P15 (18) (Fig. 1A). This *Bam*HI fragment was excised out of PAC 215P15 and microinjected into fertilized mouse oocytes. Four founders out of a total of 62 potential transgenic animals were obtained. One of these died prematurely and a second failed to transmit the transgene to her offspring. The remaining two were bred to C57Bl 6J $Smn^{+/-}$ mice to produce $Smn^{+/-}$;*SMN2* progeny. The copy number of the transgenes was estimated by Southern blotting of DNA isolated from tail tissue. Based on densitometric analysis, hemizygous mice from lines 89 and 566 carry one and eight *SMN2* copies, respectively (Fig. 1B).

The early embryonic lethality seen in *Smn* knockout mice created by Schrank *et al.* (14) probably results when maternal SMN is depleted. To test whether the *SMN2* transgene could rescue this embryonic lethality and produce a mouse with SMA, we initially mated our transgenic mice with $Smn^{+/-}$ mice. F_1 offspring carrying an *Smn* knockout allele and the *SMN2* transgene were then interbred to produce F_2 progeny. Approximately 15% (46/312) of the F_2 progeny derived from the low copy founder, 89, were either stillborn or died within 6 h of birth (Table 1). The vast majority (42/46) of these mice were shown by genotyping to carry one or two copies of the transgene and be homozygous for the *Smn* knockout allele ($Smn^{-/-}$;*SMN2*). In fewer cases (14/312), $Smn^{-/-}$;*SMN2* pups survived for up to 6 days of age. A total of 18% (56/312) of the progeny were found to be of the genotype $Smn^{-/-}$;*SMN2*. This is slightly lower than the expected frequency (20%) for this genotype if both parents are heterozygous for *SMN2*. However, this may be explained by some death occurring *in utero*.

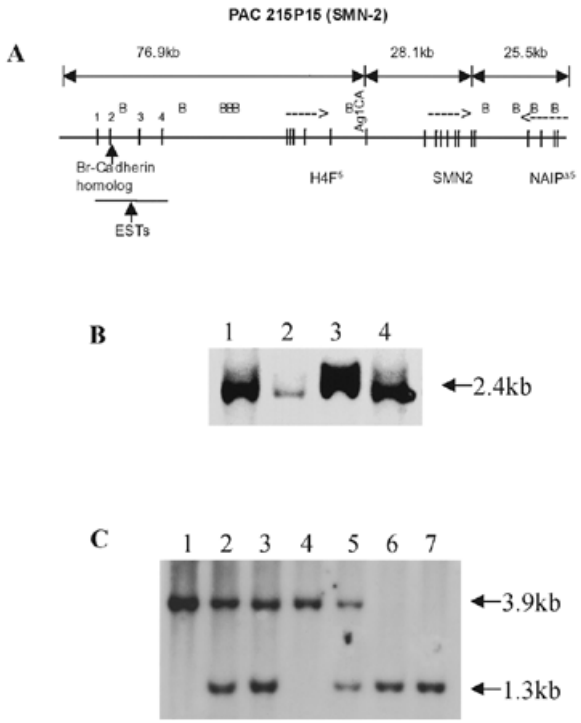

Figure 1. (**A**) Diagrammatic representation of *SMN2* lying within PAC 215P15. Also depicted are the *H4F5* gene, portions of the *NAIP* gene and exons lying 5′ to *SMN2*. B, *Bam*HI site. Broken arrows indicate direction of expression. (**B**) Southern blot analysis of *SMN2* transgenic founders and normal human controls. Lanes 1 and 4, normal human DNA carrying four copies of *SMN* (2 centromeric and 2 telomeric); lane 2, founder 89 carrying one copy of *SMN2*; lane 3, founder 566 carrying eight copies of *SMN2*. (**C**) Southern blot analysis of low and high copy *SMN2* transgenic mice showing presence/absence of mouse *SMN*. Lane 1, normal non-transgenic $Smn^{+/+}$ mouse; lanes 2 and 3, $Smn^{+/-}$;*SMN2* male and female mice, respectively; lanes 4, 5 and 6, pups of individuals in lanes 2 and 3: all were found to carry *SMN2* (data not shown); lane 7, DNA from a high copy $Smn^{-/-}$;*SMN2* 1-month-old mouse. The upper 3.9 kb band indicates an intact mouse *Smn* allele and the lower 1.3 kb band the knockout allele.

Table 1. Genotypes of $Smn^{-/-}$;*SMN2* intercross progeny

	+/+	+/–	–/–	$Smn^{-/-}$;*SMN2*	Total
	74	182	56	56	312
Died within 6 h of birth	–	4	42	42	
Died at 4–6 days of age	–	2	14	14	

+, mouse *Smn* allele; –, knockout allele.
Genotyping was done either by PCR (primers available on request) or Southern blot analysis. Every $Smn^{-/-}$ mouse was also found to carry the *SMN2* transgene.

At birth the low copy $Smn^{-/-}$;*SMN2* mice looked phenotypically normal. Mice stillborn or those that die shortly after birth were slightly smaller (average weight = 1.33 g, $n = 5$) than normal littermates (average weight = 1.51 g, $n = 5$). The stillborn mice were often cyanotic. Affected $Smn^{-/-}$;*SMN2* mice that survived for 4–6 days appeared phenotypically indistinguishable from littermates in the first 48 h after birth. This period was followed by a rapid deterioration in the next 48–72 h and death. These mice began to show decreased movement, decreased or lack of suckling and laboured breathing by 48 h postnatal. By 72–96 h after birth the mice showed a marked phenotype including a tremor in the limbs. At this stage $Smn^{-/-}$;*SMN2* mice could be readily distinguished from

Figure 2. A 4-day-old low copy *Smn*$^{-/-}$;*SMN2* pup lying next to a normal littermate carrying at least one normal *Smn* allele. Typical phenotypic characteristics of SMA mice at this stage include decreased movement, weakness and an inability to right themselves.

their normal littermates (Fig. 2). Removing some unaffected littermates at 3 days of age to prevent competition for food and warmth did not increase survival of *Smn*$^{-/-}$;*SMN2* mice. When placed on their side, affected mice had difficulty righting themselves. Some *Smn*$^{-/-}$;*SMN2* also had a bell-shaped trunk, likely attributable to intercostal muscle weakness, a characteristic feature of type I SMA (24). *Smn*$^{-/-}$;*SMN2* mice died 4–6 days after birth by which time they were considerably smaller (average weight = 1.47 g, n = 4) than littermates (average weight = 4.59 g, n = 5) positive for murine *Smn*.

A similar breeding strategy was followed with mice derived from the high copy number transgene, 566. At birth *Smn*$^{-/-}$ mice carrying the high copy transgene did not show any obvious phenotype. At the time of going to press two of these mice are 10 months old and still do not display any overt weakness. The only unusual phenotypic feature of these mice is a short, thick tail. This becomes evident at ~2 weeks of age; however, we are unsure of the significance of this to SMA pathology. It is therefore clear that the *SMN2* gene is capable of rescuing the embryonic lethality of the *Smn*$^{-/-}$ knockout. Our results demonstrate that an increased copy number of the *SMN2* gene greatly reduces the severity of the phenotype seen in the low copy transgenic mice.

Expression of SMN2 in transgenic animals. In humans, the *SMN* genes have been shown to be ubiquitously expressed. In addition, it has been shown that there are several alternatively spliced isoforms of *SMN* (25). The *SMN2* gene produces all four isoforms, the predominant form being that lacking exon 7. To examine *SMN2* transgene expression, 1–2 µg of poly(A)$^+$ RNA, obtained from brain, liver and spinal cord of 4-day-old *Smn*$^{-/-}$ mice carrying the low copy transgene and adult *Smn*$^{-/-}$ individuals carrying the high copy transgene, was hybridized at high stringency with an *SMN* cDNA probe spanning exons 6–8. The *SMN2* transgenes were ubiquitously expressed in all tissues examined (Fig. 3A). This is similar to the observed expression pattern for endogenous *SMN2*. Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) using primers in exons 4 and 8 also showed that the *SMN2* transgenes in both

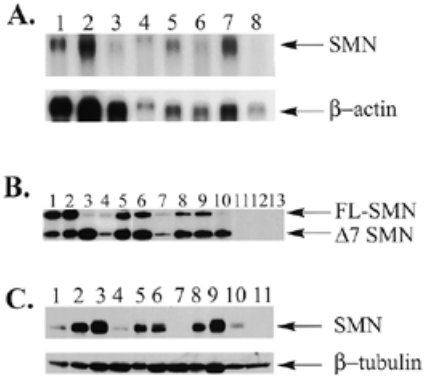

Figure 3. (**A**) Northern blot analysis of tissue from the low and high copy *Smn*$^{-/-}$;*SMN2* transgenic mice. Lane 1, RNA from spinal cord tissue of a type I SMA patient; lanes 2, 3 and 4, RNA from liver, brain and spinal cord, respectively, of a low copy *Smn*$^{-/-}$;*SMN2* mouse; lanes 5, 6 and 7, RNA from liver, brain and spinal cord, respectively, of a high copy *Smn*$^{-/-}$;*SMN2* mouse; lane 8, RNA from liver tissue of a non-transgenic *Smn*$^{+/+}$ mouse. Also shown is the same blot probed with a β-actin cDNA probe. (**B**) RT–PCR on tissue from low and high copy *Smn*$^{-/-}$;*SMN2* mice and appropriate controls using the exon 4 forward primer (5′-GTGAGAACTCCAG-GTCTCCTGG-3′) and the exon 8 reverse primer (5′-CTAC-AACACCCTTCTCACAG-3′). Although all four alternatively spliced isoforms are expressed by the *SMN2* transgenes, only the two major isoforms are shown. Lanes 1 and 2, liver and spinal cord, respectively, from normal human tissue; lanes 3, 4 and 5, lymphocytes, liver and spinal cord, respectively, from an SMA patient; lanes 6 and 7, brain and liver, respectively, from a 4-day-old high copy *Smn*$^{-/-}$;*SMN2* transgenic mouse; lanes 8 and 9, brain and liver, respectively, from a 4-day-old low copy *Smn*$^{-/-}$;*SMN2* mouse; lane 10, brain from a 1-day-old low copy *Smn*$^{-/-}$;*SMN2* mouse; lanes 11 and 12, brain and liver, respectively, from a non-transgenic *Smn*$^{+/+}$ mouse; lane 13, blank control. Note the difference in ratios between full-length *SMN* and the isoform lacking exon 7 in normals, SMA patients and SMA mice. Also note that the levels of alternatively spliced isoforms are not exactly the same in different human tissues. (**C**) Western blot analysis using MANSMA2 on brain tissue of embryonic day 15, 1- and 4-day-old high and low copy *Smn*$^{-/-}$;*SMN2* transgenic mice and normal controls. Lanes 1, 2 and 3, embryonic day 15 low copy *Smn*$^{-/-}$;*SMN2*, high copy *Smn*$^{-/-}$;*SMN2* and *Smn*$^{+/+}$ mice, respectively; lanes 4, 5 and 6, tissue from 1-day-old mice with similar genotypes to those in lanes 1, 2 and 3, respectively; lanes 7, 8 and 9, tissue from 4-day-old mice with similar genotypes to those in lanes 1, 2 and 3, repectively; lanes 10 and 11, normal human and type I SMA spinal cord tissue. Also shown is the blot probed with an antibody for β-tubulin.

lines 89 and 566 produce the expected size transcripts in the correct ratios (Fig. 3B). Like endogenously expressed human *SMN2*, most of the transcripts from the transgenes lacked exon 7.

To examine the expression of the *SMN* protein from the *SMN2* transgenes, liver (data not shown) and brain tissue from low copy *Smn*$^{-/-}$;*SMN2*, high copy *Smn*$^{-/-}$;*SMN2* and normal controls was used for western blot analysis. Our results clearly show that low copy *Smn*$^{-/-}$;*SMN2* mice have vastly reduced levels of the SMN protein (Fig. 3C). Compared with age-matched controls, these affected mice express 10- to 20-fold less SMN protein. This is consistent with previous reports of comparisons between normal human individuals and type I SMA patients (10,11). It is also interesting to note that there is a pronounced decrease in SMN protein levels in the low copy *Smn*$^{-/-}$;*SMN2* mice with the progression of age from embryonic day 15 to postnatal day 4. In control mice there was a similar although less pronounced change in SMN levels. A change in SMN levels during development has also been reported in humans (26). These results and our current observations indicate that SMN expression is developmentally regulated. A comparison of the high copy *Smn*$^{-/-}$;*SMN2* transgenic mice with controls shows that they produce equivalent levels of

Table 2. Motor neuron counts of the facial nucleus and spinal cord (L1–L6) in high and low copy *Smn*$^{-/-}$;*SMN2* mice and normal controls carrying two intact mouse *Smn* alleles

	Low copy *Smn*$^{-/-}$;*SMN2*	High copy *Smn*$^{-/-}$;*SMN2*	*Smn*$^{+/+}$;*SMN2*
Facial motor neurons			
P1	3936 ± 95 (n = 5)	4002 ± 641 (n = 2)	4105 ± 390 (n = 3)
P3–5	1709 ± 189 (n = 4)	2617 ± 173 (n = 4)	2981 ± 152 (n = 4)
Spinal motor neurons			
P1	1876 ± 143 (n = 6)	2186 ± 83 (n = 2)	2299 ± 290 (n = 2)
P3–5	1113 ± 36 (n = 4)	1614 ± 85 (n = 4)	1740 ± 123 (n = 3)

P1, postnatal day 1; P3–5, postnatal days 3–5.
One-way ANOVA with Bonferroni's multiple comparison test revealed statistically significant differences in motor neuron numbers on P3–5 mice. The number of facial and spinal motor neurons in the low copy *Smn*$^{-/-}$;*SMN2* transgenic animals was significantly lower than in the other two control groups ($P < 0.05$). The difference in facial motor neurons on P3–5 between the high copy *Smn*$^{-/-}$;*SMN2* mice and *Smn*$^{+/+}$;*SMN2* animals was not statistically significant.

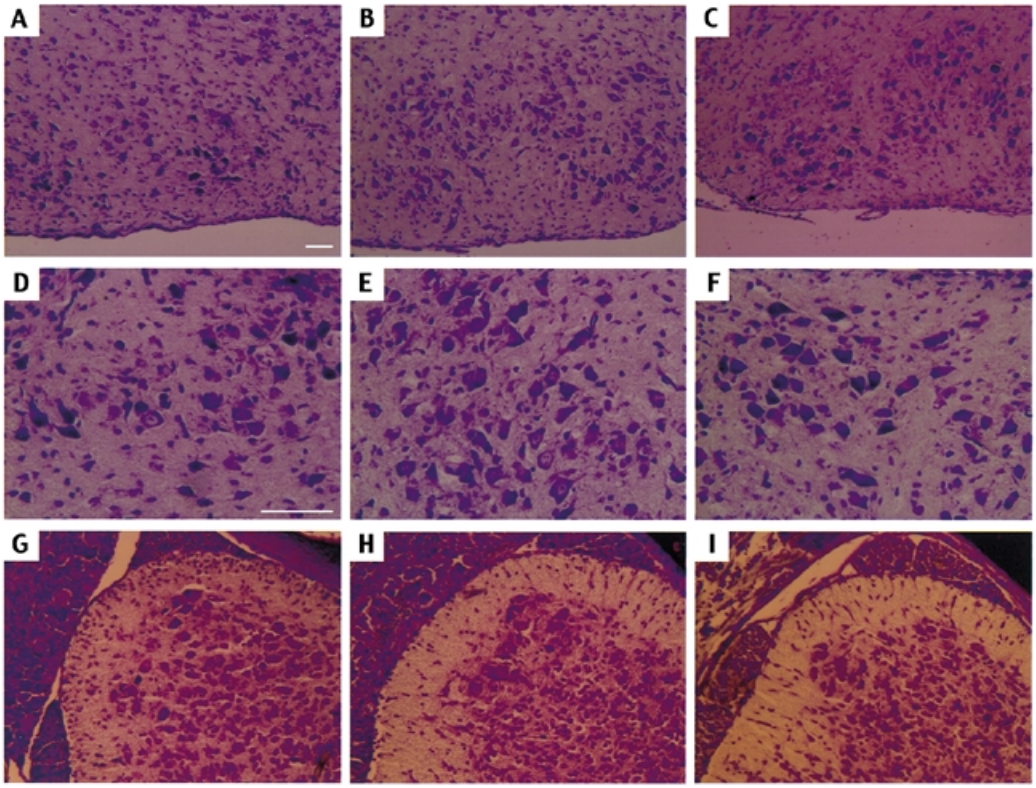

Figure 4. Histological view of facial and spinal motor neurons of high and low copy *Smn*$^{-/-}$;*SMN2* mice and normal controls. (**A**, **D** and **G**) Facial (A and D) and spinal (G) motor neurons in 5-day-old low copy *Smn*$^{-/-}$;*SMN2* transgenic animals; (**B**, **E** and **H**) facial (B and E) and spinal (H) motor neurons of high copy *Smn*$^{-/-}$;*SMN2* transgenic mice; (**C**, **F** and **I**) facial (C and F) and spinal (I) motor neurons from *Smn*$^{+/+}$;*SMN2* mice. Scale bar in (A) also applies to (B, C, G, H and I) and scale bar in (D) also applies to (E and F): 100 μm.

SMN protein. If, as suggested in earlier reports (10,11), SMA disease severity is modulated by SMN protein levels, then it is not surprising that the high copy *Smn*$^{-/-}$;*SMN2* transgenic mice are phenotypically normal.

Severe loss of motor neurons in low copy Smn$^{-/-}$*;SMN2.* One of the most striking neuropathological features of infantile SMA is the degeneration of the motor neurons of the spinal cord and the lower brain stem. However, the timing of this motor neuron death in SMA is unknown. To determine whether our mice, in particular the low copy *Smn*$^{-/-}$;*SMN2* ones, suffer a loss of these motor neurons, animals between the ages of 1 and 5 days were sacrificed and perfused, and their motor neurons in the spinal cord and facial nucleus counted in paraffin-embedded serial sections. Interestingly, we found relatively normal numbers of motor neurons in the spinal cord and facial nucleus of 1-day-old high and low copy *Smn*$^{-/-}$; *SMN2* mice compared with age-matched controls (Table 2). However, by postnatal day 5, there was a dramatic loss of these cells in the low copy *Smn*$^{-/-}$;*SMN2* mice. This degeneration varied from an ~35% loss in the spinal cord to a 40% loss in the facial nucleus.

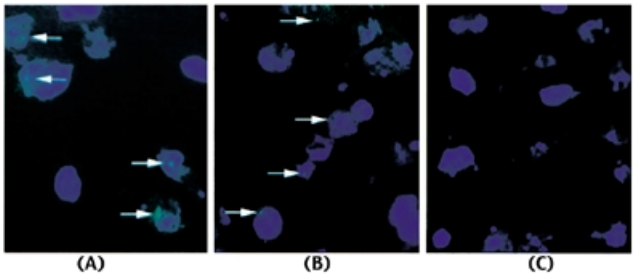

Figure 5. Immunocytochemical staining of SMN in spinal motor neurons of 5-day-old high copy *Smn*$^{-/-}$;*SMN2* (**A**), *Smn*$^{+/+}$ (**B**) and low copy *Smn*$^{-/-}$;*SMN2* (**C**) mice showing brightly staining nuclear gems (arrows) and the diffuse cytoplasmic SMN in the high copy *Smn*$^{-/-}$;*SMN2* mice and normal *Smn*$^{+/+}$ control. There is almost no SMN in spinal cord of the low copy *Smn*$^{-/-}$;*SMN2* mouse.

In addition, increased numbers of apparently dying cells with pyknotic nuclei were evident in the spinal cords and facial nuclei of these mice (Fig. 4A, D and G). This indicates that motor neurons develop normally in SMN-deficient mice and undergo death in the latter stages of the disease. The loss of motor neurons in the older low copy *Smn*$^{-/-}$;*SMN2* mice parallels the appearance of the disease phenotype. This loss begins at postnatal day 3. However, subtle degenerative changes in the motor neurons may begin prior to this since most of our low copy *Smn*$^{-/-}$;*SMN2* mice were stillborn or died very shortly after birth. In *Smn*$^{-/-}$;*SMN2* mice that survived for 4–6 days, the motor neurons apparently reached a crisis stage ~3 days after birth and were rapidly lost thereafter.

A second characteristic feature of SMA patients is the presence of atrophic muscle fibers. We examined the quadriceps and gastrocnemius muscle of 4- to 5-day-old low copy *Smn*$^{-/-}$;*SMN2* mice but did not find any remarkable pathology (data not shown). This may be because muscle in very young rodents undergoes polyneuronal innervation which is lost at ~2 weeks of age (27 and references therein). Loss of motor neurons may, therefore, begin to occur before shrinkage of motor unit size leads to denervated muscle. An examination of the gastrocnemius and quadriceps muscles from the high copy *Smn*$^{-/-}$;*SMN2* mice showed no pathology even at the age of 2 months.

Absence of gems in low copy Smn$^{-/-}$*;SMN2 mice.* Finally we sought to examine *SMN2* expression in the spinal motor neurons of our transgenic mice by immunohistochemical methods. It has been shown previously that SMN localizes to subnuclear structures termed gems. Further, it has been reported that there is a high correlation between clinical severity of the SMA phenotype and the number of gems in patient fibroblasts and spinal cord. To determine whether this is the case in our *Smn*$^{-/-}$;*SMN2* transgenic mice, lumbar spinal cord sections from 5-day-old animals were stained with anti-SMN monoclonal antibodies and visualized by fluorescence microscopy. Neonatal mice had reduced cytoplasmic staining as well as less intense and smaller gems than adult mice. It is clear from our results that the low copy *Smn*$^{-/-}$;*SMN2* mice had almost no gems in the spinal motor neurons compared with the high copy *Smn*$^{-/-}$;*SMN2* animals and normal controls (Fig. 5). Fibroblasts derived from the low copy *Smn*$^{-/-}$;*SMN2* also showed no gems (data not shown). This is similar to observations of fibroblasts and spinal cord sections from type I SMA patients and re-inforces our view that the low copy *Smn*$^{-/-}$;*SMN2* transgenic mice serve as a suitable animal model of severe infantile SMA. Moreover, unlike previously described animal models of human SMA (28,29), ours is the first that involves alterations in the mouse homolog of the *SMN1* gene and low levels of the SMN protein.

DISCUSSION

The presence of at least one intact *SMN2* gene in SMA patients and the early embryonic lethality in *Smn*$^{-/-}$ mice strongly suggested that low SMN levels are essential for development. Here we provide the first direct evidence that the *SMN2* gene can rescue the embryonic lethality in *Smn*$^{-/-}$ mice and allow development to progress to term. Our study shows that low levels of SMN protein, such as those found in type I SMA patients, are sufficient for embryonic development. Motor neuron cells which are particularly sensitive to low levels of SMN produced by the *SMN2* gene are lost in the postnatal period. This includes spinal motor neurons as well as those in the facial nucleus. Loss of the latter has not been reported in the literature in studies of human type I SMA patients. Perhaps this is due to the fact that these studies are based on limited numbers of patients that were either clinically poorly characterized or heterogeneous in presentation (27). It would not be surprising to find loss of this subset of motor neurons in SMA patients if a detailed analysis including morphometric counts was carried out. We would also note that although specific motor neuron counts have not been carried out on human type I SMA patients of different ages, it is quite likely that motor neuron death in these individuals occurs at similar points in development as that observed in our low copy *Smn*$^{-/-}$; *SMN2* mice. The fact that the disease often presents with such sudden onset after relatively normal development (27) strengthens this view.

We have shown using our high copy *Smn*$^{-/-}$;*SMN2* transgenic mice that an increased expression of the SMN protein, even if it is from the *SMN2* gene, is capable of rescuing the SMA phenotype. This result is consistent with the observation that in human SMA there is a correlation between phenotypic severity and *SMN2* copy number and supports the hypothesis that the *SMN2* gene acts as a modifier of phenotype (30,31). In addition there is a report of a very mildly affected 70-year-old SMA patient who has eight copies of the *SMN2* gene (32). This also supports our data regarding the importance of SMN dosage in determining phenotypic severity.

In summary, we have developed a mouse model whose disease symptoms and neuropathology are remarkably similar to those of severe type I SMA in humans. We have shown that motor neuron loss in low copy *Smn*$^{-/-}$;*SMN2* mice occurs postnatally and that increased *SMN2* copy number rescues all overt features of type I SMA. These results suggest that increased expression from the *SMN2* gene during postnatal development, before complete onset of the disease, will rescue the loss of motor neurons in SMA and decrease phenotypic severity. Our low copy *Smn*$^{-/-}$;*SMN2* mouse is the first true animal model of human SMA and will be extremely useful in testing possible candidates for the upregulation of the centromeric gene.

MATERIALS AND METHODS

Microinjections and genotyping of mice. PAC 215P15 was digested with *Bam*HI and *Cla*I. *Cla*I serves to digest a 29 kb band that contaminates *SMN2*. The digested DNA was run on a 0.4%

low-melt agarose gel and the 35.5 kb fragment excised out. Following purification it was resuspended in injection buffer (10 mM Tris–Cl, 0.1 M EDTA, 100 mM NaCl, 70 µM spermidine and 30 µM spermine) at a concentration of 2 ng/µl, dialyzed using a 0.05 µM membrane filter (Millipore, Bedford, MA) and then injected into fertilized FVB mouse oocytes. Copy number was assessed by Southern blot analysis of 10 µg of DNA digested with *Pst*I and probed with an ~450 bp exon 6–8 human *SMN* cDNA fragment. Digested DNA was electrophoresed on a 1% agarose gel before being transferred onto Hybond N^+ (Amersham, Piscataway, NJ). The probe was radioactively labeled and hybridized to the filter according to standard methods (33). The filter was washed at high stringency and then exposed to Hyperfilm MP (Amersham) with an intensifying screen. Densitometric analysis was performed on a Shimadzu 9000 CS scanner (Shimadzu, Kyoto, Japan).

Northern analysis and RT–PCR. Mice were sacrificed by cervical dislocation and poly(A)$^+$ RNA isolated using the QuickPrep Micro mRNA Purification kit (Amersham) according to the manufacturer's recommendations. For northern analysis, 1–2 µg of RNA was fractionated on a 1.2% agarose–formaldehyde gel prior to blotting. The filter was prehybridized and hybridized in ExpressHyb hybridization solution (Clontech, Palo Alto, CA). It was then washed as per the manufacturer's instructions. The probe used was the same as the one used for Southern analysis. The membrane was stripped after the first hybridization and reprobed with a 245 bp mouse β-actin cDNA fragment to control for loading amounts. RT–PCR was carried out on 0.2 µg of first strand cDNA. Both primers were end-labeled with [γ-^{32}P]ATP and amplification performed in a 25 µl volume containing 0.5 mM dNTPs, 1 U *taq* polymerase, 30 ng of each of the primers and 2.5 mM Mg^{2+}. Cycling conditions were: 95°C for 5 min; 20 cycles of 95°C for 1 min, 55°C for 2 min, 72°C for 3 min; with a final extension at 72°C for 8 min. Products were separated for 12 000 Vh on a 6% denaturing polyacrylamide gel which was dried and exposed to Hyperfilm-MP for 1–4 days.

Western blot analysis. Tissue (100 mg) was dissolved in blending buffer (10% SDS, 62.5 mM Tris pH 6.8, 5 mM EDTA). The concentration of the protein was determined using the BCA kit (Pierce, Rockford, IL). Protein (25–50 µg) was mixed with an equal volume of sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 10% 2-mercaptoethanol, 0.4 mg of bromophenol blue) and electrophoresed on a 12.5% polyacrylamide gel. Samples were transferred to Immobilon-P (Millipore) as previously described (11). The blot was blocked in 5% milk powder, 0.5% bovine serum albumin (BSA) in TBS–Tween (TBS-T) for 2 h, then incubated for 1 h with an anti-SMN primary antibody (MANSMA2, specific for either exon 2A or 2B) dissolved in 1% milk, 0.1% BSA in TBS-T at a 1:1000 dilution. After four washes in TBS-T at room temperature (10 min each) the blot was incubated with a goat anti-mouse HRP-linked F(ab′)$_2$ antibody (Jackson Immunoresearch, West Grove, PA) at a 1:8000 dilution. The blot was again washed four times in TBS-T and then visualized using ECL chemiluminescent reagent (Amersham) according to the manufacturer's recommendations. The blots were then stripped and reprobed with a β-tubulin antibody (Sigma, St Louis, MO) to control for loading amounts.

Histology and immunocytochemistry. One- to five-day-old low and high copy *Smn*$^{-/-}$; *SMN2* mice and normal controls were sacrificed and perfused with fresh 4% paraformaldehyde in phosphate-buffered saline (PBS). Tissue from these mice was embedded in paraffin according to conventional procedures. Serial sections (7 µm) prepared with an automated Leica (Nusloch, Germany) rotation microtome were mounted on glass slides and subjected to Nissl's staining as previously described (34). Neurons within the facial nucleus and the lumbar motor column (L1–L6) were counted and tallied after correcting for double counts of split nucleoli. Whole spinal columns were mounted on wooden blocks and flash frozen in isopentane chilled to –150°C in liquid nitrogen. Sections (8 µm) were cut, mounted on superfrost slides (Fisher Scientific, Pittsburgh, PA) and air dried for 30 min. They were then rehydrated in PBS (5 min), fixed in 4% paraformaldehyde (1 min), briefly rinsed in PBS and finally treated with ice-cold acetone (5 min). Then they were air dried (30 min) and incubated with a 1:5 dilution of mouse blocker from the MOM kit (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. Sections were then rinsed twice in PBS, blocked with 10% normal donkey serum (Jackson Immunoresearch) for 30 min and then incubated for 2 h with a mixture of anti-SMN antibodies MANSMA7, MANSMA6 and 8B3, diluted 1:100 in 1% donkey serum. After washing in PBS (three times, 5 min each) sections were incubated for 30 min with FITC-conjugated donkey anti-mouse F(ab′)$_2$ antibody (Jackson Immunoresearch) at a 1:50 dilution in PBS. Sections were washed again, mounted with 25 µl of Vectorshield (Vector Laboratories) containing a 1:6 dilution of DAPI and examined using a Nikon E800 microscope equipped with HiQ FITC and TRITC/DAPI dual wavelength filter sets (Chroma Technology, Battleboro, VT). Black and white images were captured with an Olympix Ultrapix digital camera (Olympus America, Melville, NY), pseudocolored and merged using the Olympus software.

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