

A Cell System with Targeted Disruption of the SMN Gene

FUNCTIONAL CONSERVATION OF THE SMN PROTEIN AND DEPENDENCE OF Gemin2 ON SMN*

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The motor neuron degenerative disease spinal muscular atrophy is caused by reduced expression of the survival motor neuron (SMN) protein. Here we report a genetic system developed in the chicken pre-B cell line DT40, in which the endogenous SMN gene is disrupted by homologous recombination, and SMN protein is expressed from a chicken SMN cDNA under control of a tetracycline (tet)-repressible promoter. Addition of tet results in depletion of SMN protein and consequent cell death, which directly demonstrates that SMN is required for cell viability. The tet-induced lethality can be rescued by expression of human SMN, indicating that the function of SMN is highly conserved between the two species. Cells expressing low levels of SMN display slow growth proportional to the amount of SMN they contain. Interestingly, the level of the SMN-interacting protein Gemin2 decreases significantly following depletion of SMN, supporting the conclusion that SMN and Gemin2 form a stable complex *in vivo*. This system provides a powerful setting for studying the function of SMN *in vivo* and for screening for potential therapeutics for spinal muscular atrophy.

Spinal muscular atrophy (SMA),¹ an autosomal recessive disease with characteristics of motor neuron degeneration and muscle atrophy, is a common childhood genetic disorder and the most frequent genetic cause of infant mortality (1–3). Based on the age of onset and the severity of the disease, SMA is clinically classified as the severe type I (Werdnig-Hoffman disease), the moderate type II, and the mild type III (Kugelberg-Welander disease). The survival motor neuron (*SMN*) gene has been established as the disease gene of SMA. The human genome contains two copies of the *SMN* gene because of an inverted duplication at 5q13. This phenomenon appears to be human-specific, because all other organisms examined to date have a single copy of *SMN*. Deletions or mutations of the telomeric *SMN1* gene, which result in reduced SMN protein

level, have been found in the vast majority of SMA patients (4–11).

Although motor neurons seem to be the only known cell type that is affected in SMA patients, SMN protein is expressed ubiquitously in all tissues and cell types examined (8, 12, 13). The amino acid sequence of SMN does not share significant homology with any protein with a known function; nor does it contain any domains of known function. Although several lines of evidence have suggested that SMN participates in several divergent cellular processes, the question of how reduction of the SMN level leads to motor neuron degeneration remains open. In addition to its cytoplasmic localization, SMN is found in a novel subnuclear structure, named gems, which are found in the vicinity of, and often overlap with, coiled bodies (14). The function of coiled bodies is unknown, but they contain spliceosomal snRNPs (small nuclear ribonucleoprotein particles), which function in pre-mRNA splicing, and components of small nucleolar ribonucleoprotein particles, which are involved in pre-rRNA processing. This has led to the speculation that coiled bodies may play some roles in snRNP and small nucleolar ribonucleoprotein particle metabolism (15). The fact that gems and coiled bodies are often associated and contain similar sets of proteins and RNAs suggests that they have related functions. In line with this idea, SMN has been shown to interact with a group of Sm proteins, the core proteins of snRNPs, and a novel protein, Gemin2 (formerly known as SIP1), both *in vitro* and *in vivo* (14). Injection of antibodies against either SMN or Gemin2 into *Xenopus* oocytes inhibits assembly and import of snRNPs, suggesting that the SMN-Gemin2 complex performs an important function in snRNP metabolism (16, 17). A dominant negative mutant of SMN, SMN Δ N27, also inhibits snRNP assembly in the cytoplasm (18). Moreover, the nuclear pool of SMN protein was found to be required for pre-mRNA splicing, probably by facilitating regeneration or recycling of snRNPs in the nucleus (18). Recently, two additional proteins, Gemin3 and Gemin4, that are associated with SMN have been described (19, 20). SMN may also be involved in regulation of gene expression by interacting with transcriptional activators (21–23). The ability of SMN to directly bind RNA, along with its close localization to microtubules in the cytoplasm and neuronal dendrites and axons, raises a possibility that SMN is involved in the transport of RNA (24–27).

SMN is evolutionarily conserved throughout eukaryotes, because homologues of SMN have been identified in many organisms. Genetic studies have shown that *SMN* is an essential gene in mice, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* (28–32). Mice carrying the human centromeric *SMN2* transgene under an *SMN* null background are viable and display phenotypes similar to the symptoms of SMA patients, thereby confirming that *SMN* is the disease gene of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF322650.

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¹ The abbreviations used are: SMA, spinal muscular atrophy; SMN, survival motor neuron; snRNP, small nuclear ribonucleoprotein particle; tet, tetracycline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; cSMN, chicken SMN; huSMN, human SMN; wt, wild-type; PI, propidium iodide.

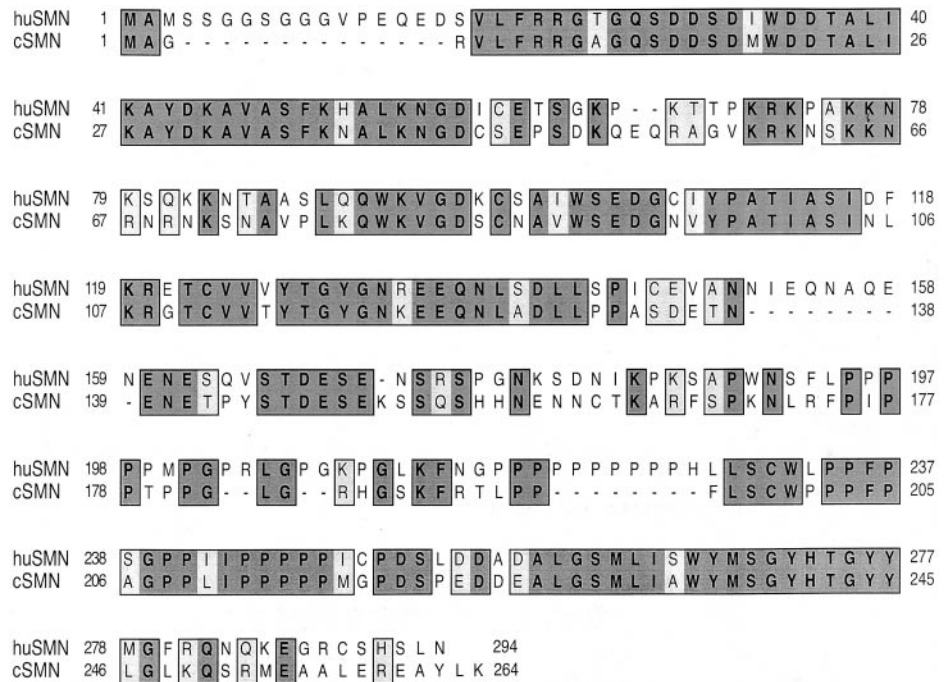


FIG. 1. Alignment of huSMN and cSMN. Amino acid sequences of huSMN and cSMN are aligned by MacVectorTM 6.5. The residues that are conserved between huSMN and cSMN are boxed. The identical amino acids are shaded dark and shown as bold, whereas similar amino acids are shaded light. The GenBankTM accession numbers of huSMN and cSMN are NM_000344 and AF322650, respectively.

SMA (33, 34). However, little progress has been made from these studies to elucidate the SMN function.

Homologous recombination occurs at exceptionally high frequencies in the chicken pre-B cell line DT40, which makes DT40 a useful genetic system for targeted gene disruption and for studying gene functions (35). Moreover, disruption of an essential gene is possible in DT40 when the gene product is expressed from a conditional promoter (36). To gain further insight into the function of SMN, we constructed a DT40 cell line in which the endogenous *SMN* gene is disrupted, and SMN protein is produced from an SMN cDNA under the control of the tetracycline (tet)-repressible promoter. Here we have used this system to show that SMN is essential for cell viability and that human SMN can functionally complement for chicken SMN, even though these two proteins are only ~60% identical in amino acid sequence. This cell line should provide a powerful system for the characterization of SMN function and for screening for potential therapeutic drugs for SMA.

MATERIALS AND METHODS

Library Screening and Plasmid Constructs—A chicken embryonic fibroblast cDNA library (Stratagene) was screened with a full-length human SMN cDNA radioactively labeled by using a random labeling kit (Amersham Pharmacia Biotech). Positive clones were recovered by *in vivo* excision according to the manufacturer's instruction. A chicken genomic library was screened with a full-length chicken SMN cDNA labeled as above. Positive clones were mapped by restriction enzyme digestions followed by Southern blotting. A genomic DNA fragment containing exons 1–5 of *cSMN* gene was fully sequenced.

A hygromycin resistance gene under the control of the chicken β -actin promoter was inserted into cSMN genomic DNA, replacing the entire exon 3 and part of exon 4 of the *cSMN* gene. To express cSMN cDNA from a tetracycline-repressible promoter, two plasmid constructs were used, Puro-tTA and 108-cSMN. Puro-tTA containing a cDNA encoding VP16-tetR chimeric protein (38) under the control of the chicken β -actin promoter, as well as a puromycin resistance gene, has been described previously (36). 108-cSMN contains a full-length cSMN cDNA placed downstream of tetO-CMV minimal promoter (38) and a histidinol resistance gene. Details of plasmid constructions are available upon request.

Expression vectors VSV-G6 and Hit-60 encoding the envelope glycoprotein of vesicular stomatitis virus and the Moloney murine leukemia virus gag-pol protein, respectively, a retroviral vector pMX, and pMX-enhanced green fluorescence protein are gifts from Dr. Paul Bates (University of Pennsylvania). To construct the retroviral expression

vectors pMX-cSMN and pMX-huSMN, *NcoI-XhoI* and *EcoRI-XhoI* DNA fragments covering the coding regions of full-length cSMN and huSMN, respectively, were inserted between *BamHI* and *XhoI* sites of pMX.

Cell Culture and Transfections—DT40 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Hyclone), 1% chicken serum (Sigma), 2 mM L-glutamine, and 50 μ M 2-mercaptoethanol. DT40 cells were transfected by electroporation as described previously (35). To express cSMN cDNA from a tetracycline-repressible promoter, DT40 cells were cotransfected with Puro-tTA and 108-cSMN and selected in medium containing puromycin and histidinol. Concentrations of antibiotics or drugs used in this study are as follows: 1.5 mg/ml hygromycin B (Calbiochem), 1 mg/ml histidinol (Sigma), 0.5 μ g/ml puromycin (Sigma). Tetracycline hydrochloride was purchased from Sigma and used as indicated.

Retrovirus Production and Infection—Transfections of HEK293T cells were performed by the calcium phosphate method according to the manufacturer's manual (CLONTECH). To package retrovirus for infection, HEK293 cells were cotransfected with 10 μ g each of VSV-G6, Hit-60, and a pMX vector containing cDNA of interest. 48 h after transfection, the culture supernatant was collected, passed through a 0.45- μ m filter, supplemented with 10 ng/ml tet, and applied to 1×10^6 S5 cells. 12 h later, the S5 cells were pelleted and resuspended in RPMI 1640 medium containing 1 μ g/ml tet.

Antibodies and Western Blotting—Recombinant His-tagged cSMN protein was used to immunize mice and rabbits to produce polyclonal antibodies against cSMN. Anti-Gemin2 monoclonal antibody 2E17 and anti-hnRNP A2 monoclonal antibody are described elsewhere (14).² The number of cells in each sample was counted. A pellet of 1×10^6 cells was lysed in 40 μ l of 2 \times SDS loading buffer. Following a brief sonication, 20 μ l of the lysate was loaded on a 12.5% SDS-PAGE gel. Western blotting was performed essentially as described (19).

Propidium Iodide Intake Assay—S5 cells were grown in medium containing 1 μ g/ml tet for 24, 48, 72, or 96 h. Control S5 cells were maintained in medium containing 10 ng/ml tet. Cells were collected, washed with 1 \times phosphate-buffered saline, and resuspended in 1 \times phosphate-buffered saline containing 5 μ g/ml propidium iodide at a concentration of 2×10^6 cells per ml. Immediately thereafter, the samples were analyzed by flow cytometry on a FACS Scan machine (Becton-Dickinson).

RESULTS

Targeted Disruption of the cSMN Gene—We cloned cSMN cDNA by screening a chicken cDNA library with huSMN cDNA as a probe. The full-length cSMN cDNA encodes a protein of

² L. Wan and G. Dreyfuss, unpublished data.

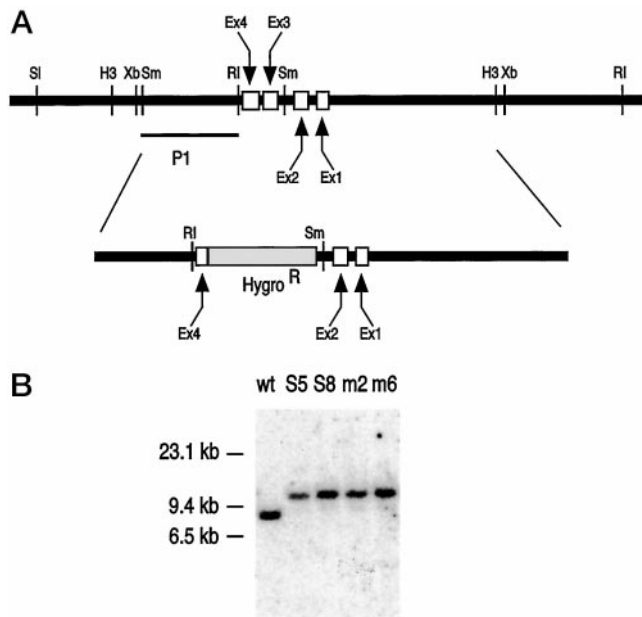


FIG. 2. Disruption of the *cSMN* gene by homologous recombination. *A*, schematic of the *cSMN* gene locus and gene targeting strategy. The positions of exons (*Ex*) 1–4 of the *cSMN* gene are indicated as *open boxes*. The hygromycin resistance gene (*Hygro^R*) is indicated as a *shaded box*. The restriction enzyme sites shown are as follows: *H3*, *HindIII*; *RI*, *EcoRI*; *SI*, *SalI*; *Sm*, *SmaI*; *Xb*, *XbaI*. The position of the probe (*P1*) used in Southern blot screening is indicated. *B*, C2 cells were transfected with Hyg-SMN and screened for homologous recombination by Southern blotting. Genomic DNAs of untransfected DT40 cells (wt) and four homologous recombinants (S5, S8, m2, and m6) were digested with *XbaI* and hybridized with the radioactively labeled probe P1. The positions of λ DNA/*HindIII* markers are indicated. *kb*, kilobases.

264 amino acids, which is 60% identical and 73% similar in sequence to the huSMN protein (Fig. 1). The *cSMN* cDNA was then used to screen a chicken genomic library to obtain genomic DNA fragments covering the entire *cSMN* locus. A targeting construct, Hyg-SMN, was made by replacing the entire exon 3 and part of exon 4 of the *cSMN* gene with a hygromycin-resistance gene (Fig. 2A). Following transfection of wild-type (wt) DT40 cells, screening of over 50 hygromycin-resistant clones by Southern blotting did not yield any homologous recombinant (data not shown). The intensity of the band on Southern blots derived from a random integration was the same as that of the band corresponding to the endogenous locus, indicating that there is only a single allele of the *cSMN* gene in DT40 cells. Although the cause of this is not known, we note that a similar phenomenon, namely that there is a single allele for the tyrosine kinase gene *syk* in DT40, has been previously reported (37).

Given that homologous recombination occurs at a frequency of over 40% in DT40, the fact that we could not obtain viable clones in which the single *cSMN* allele had been disrupted strongly suggests that *cSMN* is required for cell viability. To circumvent this problem, we first established a DT40 cell line, termed C2, which expresses *cSMN* protein from a cDNA under the control of a tet-repressible promoter (38) (see “Materials and Methods”). Addition of 1 μ g/ml tet to the culture medium completely shut off transcription of the *cSMN* cDNA. Interestingly, C2 cells maintained in a medium lacking tet displayed slow growth and cell death, indicating that overexpression of *cSMN* is deleterious.³ We noticed, however, that C2 cells maintained in a medium containing 10 ng/ml tet showed normal

³ J. Wang and G. Dreyfuss, unpublished results.

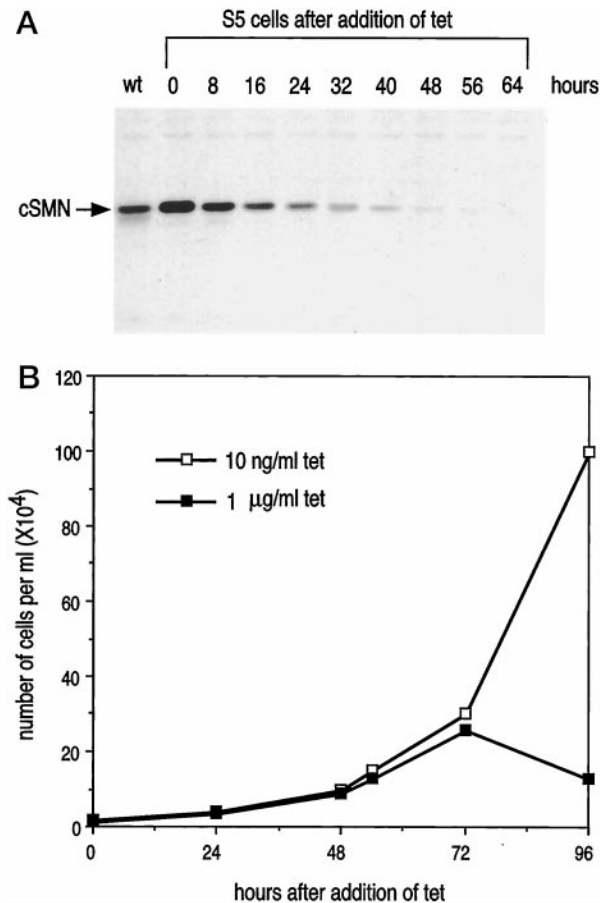


FIG. 3. *cSMN* is required for DT40 cell viability. *A*, S5 cells were grown in medium containing 1 μ g/ml tet for the time period indicated on the *top*. Lysates of these S5 cells and untransfected DT40 cells (wt) were resolved by SDS-PAGE and subjected to Western blotting using a rabbit anti-*cSMN* polyclonal antibody. The position of *cSMN* protein is indicated. *B*, an equal number of S5 cells were split into medium containing 10 ng/ml or 1 μ g/ml tet. Numbers of live cells were counted at the time point indicated. The *open* and *solid boxes* indicate the cells cultured in the presence of 10 ng/ml and 1 μ g/ml tet, respectively. The growth curve was repeated at least three times. *C*, S5 cells were treated with 1 μ g/ml tet for 24, 48, 72, or 96 h. These cells and control S5 cells were incubated with 5 μ g/ml PI and analyzed by flow cytometry. The *horizontal axis* indicates the relative intensity of PI fluorescence in a single cell. The *vertical axis* indicates the cell number. The M1 region represents the live cells that are not permeable to PI. The M2 region represents the cells that are permeable to PI and are considered dead. The percentages of M2 region are indicated.

growth and expressed high levels of exogenous *cSMN* protein (see below). Thus, knockout of the *cSMN* gene was carried out in C2 cells cultured in the presence of 10 ng/ml tet. C2 cells were transfected with linearized Hyg-SMN plasmid DNA by electroporation and selected in hygromycin-containing medium. Genomic DNAs were extracted from hygromycin-resistant clones, digested with *XbaI*, and subjected to Southern blotting using probe 1 (see Fig. 2A). Homologous recombinants should contain a longer *XbaI* fragment (~11 kilobases), because of the insertion of the hygromycin-resistant gene, and lose the wt *XbaI* fragment (~8.5 kilobases; Fig. 2A). Indeed, several independent clones showed the expected pattern on Southern blotting and thus are *cSMN*-knockout cell lines (Fig. 2B). Clone S5 was selected for further investigation and routinely maintained in a medium containing 10 ng/ml tet. These cells will be referred to hereafter as control cells unless otherwise indicated.

Depletion of *cSMN* Protein Results in Cell Death—To characterize the expression of *cSMN*, we produced mouse and rab-

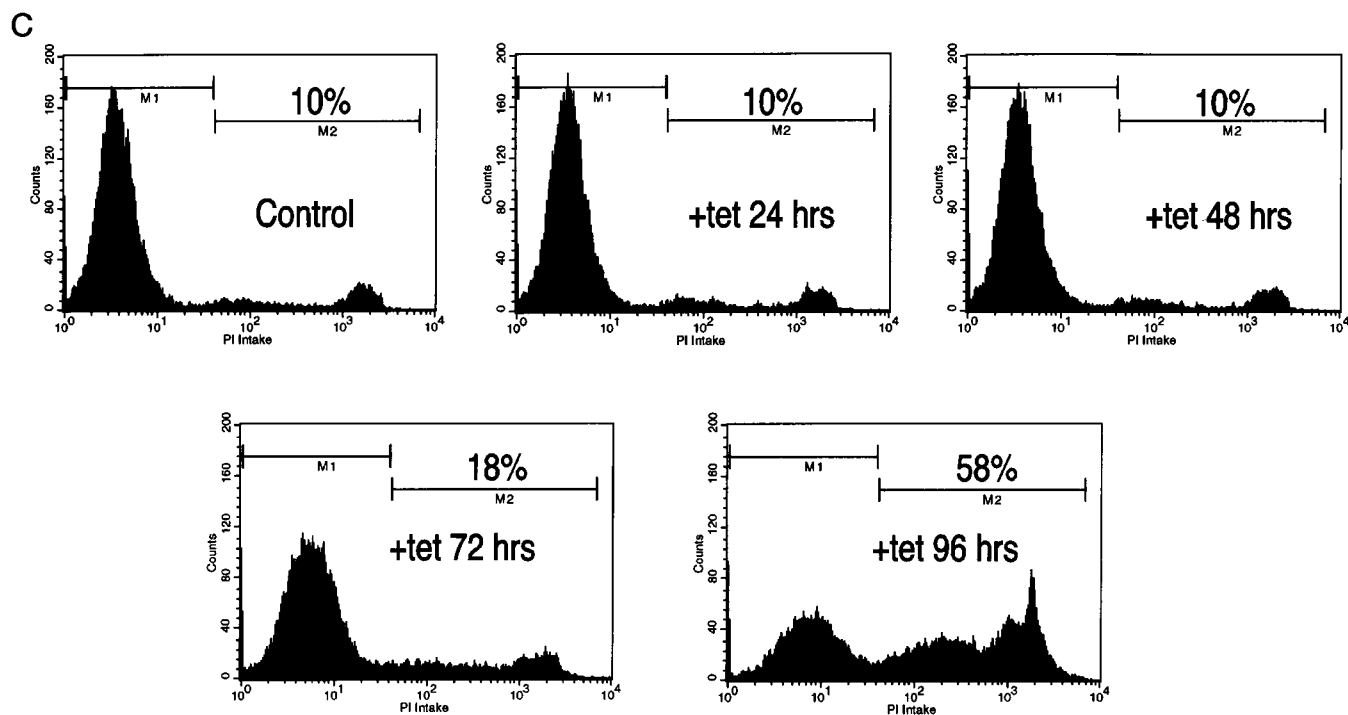


FIG. 3—continued

bit polyclonal antibodies to it. These antibodies recognized a protein band of ~38 kDa on Western blots and immunoprecipitated cSMN protein produced by transcription and translation of the cSMN cDNA in a rabbit reticulocyte lysate (data not shown). To follow the depletion of cSMN, cell samples were taken every 8 h, after S5 cells were split and placed in fresh medium containing 1 $\mu\text{g/ml}$ tet, and analyzed by Western blotting with an anti-cSMN antibody (Fig. 3A). A lysate of an equivalent number of cells was loaded on each lane. As indicated above, control S5 cells normally express a high level of cSMN, about 2–3-fold that of wt DT40 cells (Fig. 3A, first two lanes). Upon addition of tet, cSMN protein levels decreased, and after 48 h there was only a trace amount of cSMN protein in S5 cells.

To determine the effect of cSMN depletion on cell growth, we compared the growth of S5 cells in the presence of either 10 ng/ml or 1 $\mu\text{g/ml}$ tet. The number of live cells that are round and bright by phase-contrast microscopy and that are impermeable to the vital stain trypan blue was counted at the time points indicated (Fig. 3B). A propidium iodide (PI) intake assay was also performed to measure the extent of cell death (Fig. 3C). Cells that have a damaged cellular membrane are permeable to PI and are thus scored as dead. Control S5 cells double every 16 h, considerably slower than the 10-hour cell cycle of wt DT40 (data not shown). The slow growth of S5 cells is not caused by overexpression of cSMN but is rather due to expression of the trans-activator for the tet-repressible promoter. A similar effect of the tet-off system on cell growth has been reported previously (36). In the first 48 h, S5 cells maintained in a medium containing 1 $\mu\text{g/ml}$ tet showed no difference in growth rate compared with control cells. Between 48 and 72 h, the number of S5 cells with barely detectable cSMN protein doubled. The majority of these cells were still alive after 72 h, because there was only a slight increase in cell death (Fig. 3C), but massive cell death occurred between 72 and 96 h. PI intake assays showed that more than 50% of the cells were dead after 96 h (Fig. 3C). Taken together, these results indicate that cSMN is essential for DT40 cell viability.

Expression of huSMN Rescues Lethality in cSMN-depleted

Cells—The tet-induced lethality allows us to test whether a given protein can functionally replace cSMN in a complementation assay. Briefly, S5 cells were transfected with an expression vector containing a cDNA encoding the test protein and then selected in the presence of 1 $\mu\text{g/ml}$ tet. The appearance of surviving cells after 1 week indicates that the test protein can functionally substitute for cSMN and thus support cell viability. Because of the low efficiency of transfection of DT40 cells, we used a retroviral infection technique (39) to transduce cDNAs into these cells. The replication-deficient retrovirus, derived from Moloney murine leukemia virus, was packaged in HEK293 cells cotransfected with plasmids encoding the envelope glycoprotein of vesicular stomatitis virus and Moloney murine leukemia virus gag-pol proteins and a retroviral vector containing a cDNA of the test protein. We found that up to 90% of DT40 cells could be productively infected with a virus containing an enhanced green fluorescence protein cDNA, as determined by the number of cells emitting green fluorescence. As a positive control, S5 cells were infected with a virus containing the cSMN cDNA. As expected, many cells survived following tet selection. The growth pattern of the rescued cells in the presence of 1 $\mu\text{g/ml}$ tet was indistinguishable from that of control S5 cells (data not shown). A Western blot of the lysate from the rescued cells using an anti-cSMN antibody confirmed that cSMN was expressed in the presence of 1 $\mu\text{g/ml}$ tet at a level comparable with that of the control S5 cells (Fig. 4, lane 3). As a negative control, none of the S5 cells that were infected with a virus containing the enhanced green fluorescence protein cDNA survived the tet selection.

Next, we asked whether huSMN can functionally replace cSMN. To this end, S5 cells were infected with a virus containing the huSMN cDNA and incubated with 1 $\mu\text{g/ml}$ tet for 1 week. A large number of cells remained viable after the selection. These cells displayed a growth rate that was nearly identical to that of the control S5 cells. Lysates of huSMN-rescued cells were studied by Western blotting using an anti-cSMN antibody (Fig. 4, lane 4). Previous experiments have shown that our anti-cSMN antibody cross-reacts with huSMN protein (data not shown). A protein band migrating slower than cSMN

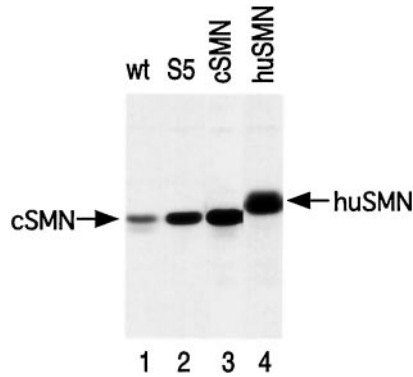


FIG. 4. Expression of huSMN can rescue cSMN-depleted cells. S5 cells were infected with a retrovirus containing either cSMN or huSMN cDNA and cultured in the presence of 1 μ g/ml tet for 1 week. Lysates of the viable cells following infection of cSMN (lane 3) or huSMN (lane 4), the control S5 cells (lane 2), and untransfected DT40 cells (wt, lane 1) were resolved by SDS-PAGE and subjected to Western blotting using a rabbit anti-cSMN polyclonal antibody. The positions of cSMN and huSMN are indicated.

specifically reacted with the antibody. The same protein band was also recognized by an anti-huSMN monoclonal antibody, 2B1 (data not shown), indicating that this band is huSMN. As expected, in the presence of 1 μ g/ml tet, the rescued cells did not express cSMN. Therefore, expression of huSMN can completely rescue cSMN-depleted cells, indicating that the function of SMN is highly conserved, if not identical, between humans and chickens.

Correlation between Cell Growth and Level of cSMN Protein—The severity of SMA closely correlates with the degree of reduction of SMN protein level in SMA patients (12, 13). We wished to determine whether there is an effect of the level of SMN on S5 cell growth. To this end, we measured growth rates of S5 cells cultured in medium containing 10, 12, 14, 16, 18, 20, or 100 ng/ml tet (Fig. 5A). To determine cSMN levels in these cells, lysates of cells grown in the respective media for 72 h were analyzed by Western blotting with an anti-cSMN antibody (Fig. 5B). In this case, the cSMN level after 72 h of treatment should reflect the steady state level in cells, because the protein is depleted after 48 h in the presence of 1 μ g/ml tet. Cells cultured in medium containing 12 and 14 ng/ml tet showed almost identical growth rates to control cells. Although cSMN levels in these cells were lower compared with control S5 cells, they were still equal to or higher than that in wt DT40 cells. A growth defect was seen in cells treated with 16 ng/ml tet, in which the cSMN level dropped to \sim 30% of that of wt DT40 cells. In the presence of 18 ng/ml tet, cell growth ceased after 72 h, and at higher concentrations of tet (e.g. 20 and 100 ng/ml), both growth arrest and cell death were apparent. Therefore, the growth rate of DT40 cells is proportional to the level of cSMN.

Significant Decrease of Gemin2 Protein following cSMN Depletion—SMN interacts avidly with Gemin2, and Gemin2 colocalizes with SMN in cells (14). Injection of anti-Gemin2 antibody into the cytoplasm of *Xenopus* oocytes inhibits assembly, maturation, and nuclear import of snRNPs (16). We asked what happens to the amount of Gemin2 following cSMN depletion. The anti-human Gemin2 monoclonal antibody 2E17 recognizes a single band of \sim 34 kDa on Western blots of total DT40 cell lysates. In addition, 2E17 coimmunoprecipitates cSMN from total DT40 lysates (data not shown). Thus, 2E17 cross-reacts with chicken Gemin2. Total lysates of S5 cells treated with 1 μ g/ml tet for 0, 24, 48, or 72 h were resolved by SDS-PAGE, and a Western blot was performed using anti-cSMN, anti-Gemin2, and, as a control for protein loadings,

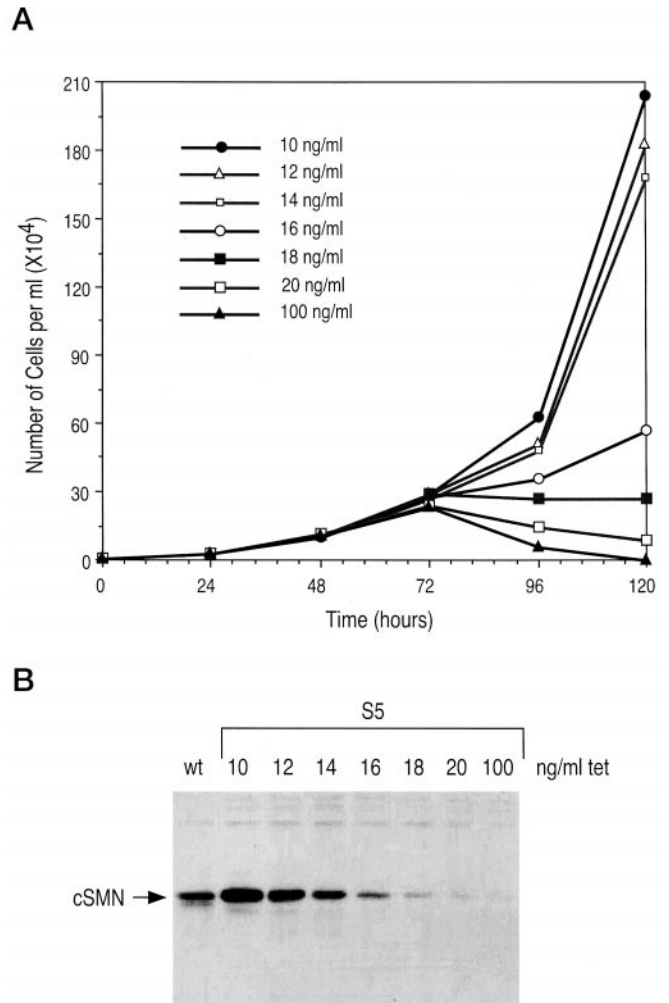


FIG. 5. Growth of S5 cells is proportional to the level of cSMN. A, after S5 cells were split into medium containing 10, 12, 14, 16, 18, 20, or 100 ng/ml tet, the number of live cells was counted every 24 h. The symbols that indicate different tet concentrations are shown in the panel. The growth curve was repeated twice. B, S5 cells were treated with tet of a concentration indicated at the top for 72 h. Lysates of these cells and untransfected DT40 cells (wt) were resolved by SDS-PAGE and subjected to Western blotting using a rabbit anti-cSMN polyclonal antibody. The position of cSMN is indicated.

anti-hnRNP A2 antibodies. As expected, cSMN was not detectable after 48 h (Fig. 6, top panel). Interestingly, in the same cells a significant decrease of the Gemin2 level was observed (Fig. 6, middle panel), and it was reduced further after 72 h. In contrast, the amount of hnRNP A2 remained unchanged in all samples (Fig. 6, bottom panel). The amounts of hnRNP C1/C2 proteins and of β -tubulin were also unchanged in all of these cells (data not shown). Therefore, depletion of cSMN results in a specific and proportional reduction of Gemin2 protein in these cells. This, along with the fact that SMN and Gemin2 associate with each other, strongly suggests that Gemin2 is stabilized when bound with SMN. Although we cannot completely rule out the possibility that SMN is involved in the regulation of the transcription, RNA processing, or translation of Gemin2 mRNA, the fact that overexpression of cSMN did not elevate the Gemin2 level (Fig. 6, middle panel, compare first two lanes) argues against this possibility.

DISCUSSION

By generating a cell line with a knockout of the *SMN* gene and conditional expression of the SMN protein, we have demonstrated directly that SMN is required for cell viability. This

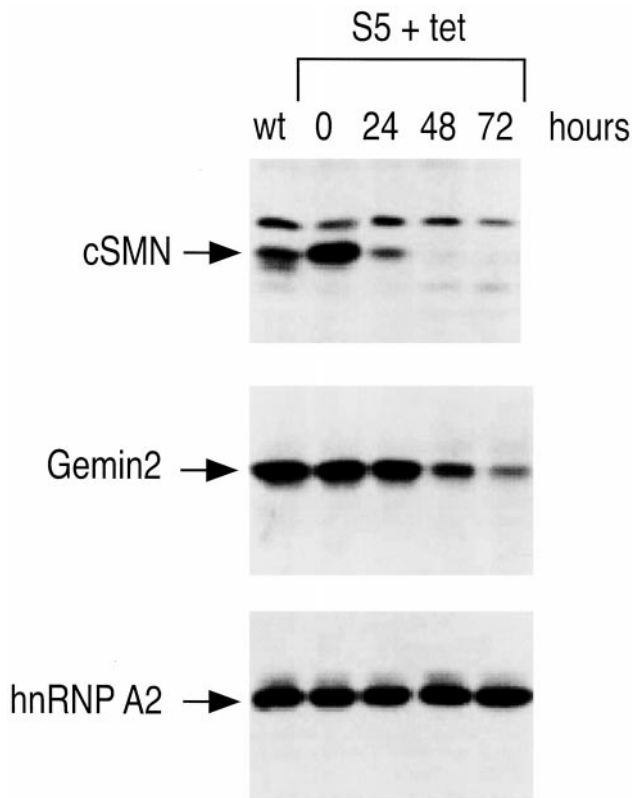


FIG. 6. Reduction of Gemin2 concomitant to cSMN depletion. S5 cells were grown in medium containing 1 μ g/ml tet for 0, 24, 48, or 72 h. Lysates of these cells and untransfected DT40 cells (wt) were resolved by SDS-PAGE and subjected to Western blotting using a rabbit anti-cSMN polyclonal antibody (top), an anti-Gemin2 monoclonal antibody 2E17 (middle), or an anti-hnRNP A2 monoclonal antibody (bottom). The positions of cSMN, Gemin2, and hnRNP A2 are indicated.

is consistent with genetic analysis of the *SMN* gene in organisms. Disruption of SMN expression in mice and *C. elegans* results in early embryonic lethality, which indicates a requirement of SMN for cell viability in embryos (28, 29). Loss of the SMN homologue in *S. pombe* also shows a lethal phenotype (30–32). Therefore, SMN is required for fundamental cellular processes that are conserved from fungi to mammals. Although the mice models for SMA generated recently (33, 34, 40) may be very useful in elucidating the pathology of SMA, the cell-based genetic system we describe here provides a setting in which the function of SMN can be studied more directly, and it offers several unique advantages. First, the SMN level in these cell lines can be modulated precisely and over a broad range, *i.e.* from none to 3–4-fold overexpression. Second, the homogeneity of a cell line should greatly facilitate characterization of phenotypes at the molecular level. More importantly, expression of huSMN protein in cSMN-depleted cells completely rescued the lethal phenotype, indicating that huSMN performs the same function as cSMN does, at least at the level of supporting cell viability and proliferation. Therefore, the function of cSMN defined by characterization of the phenotypes of cSMN-depleted S5 cells directly reflects the function of huSMN *in vivo*.

The interaction of Gemin2 and SMN has been well characterized (14). First, Gemin2 directly interacts with SMN *in vitro*. Second, Gemin2 can be coimmunoprecipitated with an SMN antibody, and *vice versa*. Finally, SMN and Gemin2 are colocalized in cells. Here we observed a significant decrease of the Gemin2 level concomitant with cSMN depletion. This confirms and extends to an *in vivo* setting the biochemically defined interaction between SMN and Gemin2 and further suggests that components of the SMN-Gemin2 complex are stabilized by

forming this complex. The effect of the SMN level on the stability of Gemin2 is reminiscent of the rapid degradation of the constituents of TFIID and SAGA complexes upon inactivation of a single TATA box-binding protein-associated factor subunit (41–45). TFIID and SAGA are well characterized complexes that function in gene transcription. It will be interesting to test whether the level of Gemin2 is also reduced in Type I SMA patients. Additional proteins that are associated with the SMN-Gemin2 complex have been reported (19, 20), and it will also be of interest to study what happens to the amounts of these proteins following SMN depletion.

The molecular basis of SMA is a reduction in the level of SMN protein. In the most severe type I SMA patients, significant decrease of SMN protein can be seen in all tissues examined (12, 13). However, motor neurons appear to be the only cells that are affected. Here we show that S5 cells that express low levels of cSMN (~30% of the protein in wt DT40 cells) display slow growth. Considering that DT40 is a pre-B cell line, this result indicates that cells other than motor neurons are also dependent on SMN and are sensitive to SMN levels. Overall growth defects can be seen in SMA mouse models (33, 34). Mice that express low levels of SMN protein and display SMA symptoms are smaller than their normal littermates after birth. Thus, it appears that human cells normally express much more SMN protein than they need, and a fraction of the SMN pool may actually be sufficient to support normal growth of cells except for motor neurons. The S5 cell line, whose growth can be specifically modulated by cSMN levels, provides a powerful system to search for potential SMA therapies. When maintained to express low levels of cSMN, S5 cells should be useful for high throughput screening for molecules that may be able to increase cell growth, presumably by enhancing the activity of cSMN, increasing its production, or slowing its turnover. Furthermore, cSMN-depleted S5 cells should be valuable for high throughput screening for compounds (if such exist) that can completely substitute for cSMN function. Given the high conservation of the SMN function, the chemical compounds that we search for will probably exert the same effect on huSMN and will, therefore, be potential therapeutic drugs for SMA. It should also be possible to carry out such screening on cSMN-depleted S5 cells whose growth is supported only by (low levels of) huSMN. Such compounds should also be useful reagents for further understanding of the normal function of SMN and the pathology of SMA.

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