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), 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, SMNΔN27, 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
Amino acid sequences of huSMN and cSMN are aligned by MacVector\textsuperscript{TM} (University of Pennsylvania). To construct the retroviral expression vectors pMX-cSMN and pMX-huSMN, NeoI-Xhol and EcoRI-Xhol DNA fragments covering the coding regions of full-length cSMN and huSMN, respectively, were inserted between BamHI and EcoRI sites of pMX. 

**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 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.

**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 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 GenBank\textsuperscript{TM} accession numbers of huSMN and cSMN are NM_000344 and AF322650, respectively.
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 one 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 μg/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. We noticed, however, that C2 cells maintained in a medium containing 10 ng/ml tet displayed normal 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-

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3 J. Wang and G. Dreyfuss, unpublished results.
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 µg/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 µg/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 µg/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 µg/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 µg/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 µg/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 µg/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-huSMN 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
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 μg/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. 5A). 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 μg/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, 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 ~34 kDa on Western blots of total DT40 cell lysates. In addition, 2E17 communoprecipitates cSMN from total DT40 lysates (data not shown). Thus, 2E17 cross-reacts with chicken Gemin2. Total lysates of S5 cells treated with 1 μg/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, 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
S5 cells were grown in medium containing 1 μg/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.

**Fig. 6. Reduction of Gemin2 concomitant to cSMN depletion.** S5 cells were grown in medium containing 1 μg/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 of interest to study what happens to the amounts of 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 at 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 conservasion 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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A Cell System with Targeted Disruption of the SMNGene: FUNCTIONAL
CONSERVATION OF THE SMN PROTEIN AND DEPENDENCE OF Gemin2 ON
SMN

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