© 2001 Oxford University Press
Human Molecular Genetics, 2001, Vol. 10, No. 24 2841–2849

Aclarubicin treatment restores SMN levels to cells derived from type I spinal muscular atrophy patients

Catia Andreassi[1], Jill Jarecki[4], Jianhua Zhou[5], Daniel D. Coovert[1], Umrao R. Monani[2], Xiaocum Chen[4], Mike Whitney[4], Brian Pollok[4], Minlei Zhang[5], Elliot Androphy[5] and Arthur H.M. Burghes[1,2,3,*]

[1]Department of Molecular and Cellular Biochemistry, [2]Department of Neurology College of Medicine and [3]Department of Molecular Genetics College of Biological Sciences, Ohio State University, Columbus, OH 43210, USA, [4]Aurora Biosciences Corporation, 11010 Torreyana Road, San Diego, CA 92121, USA and [5]Department of Dermatology, New England Medical Center and Tufts University School of Medicine, Boston, MA 02111, USA

Received August 20, 2001; Revised and Accepted September 14, 2001

Proximal spinal muscular atrophy (SMA) is a common motor neuron disorder caused by mutation of the telomeric survival of motor neuron gene *SMN1*. The centromeric survival of motor neuron *SMN2* gene is retained in all SMA patients but does not produce sufficient SMN protein to prevent the development of clinical symptoms. The *SMN1* and *SMN2* genes differ functionally by a single nucleotide change. This change affects the efficiency with which exon 7 is incorporated into the mRNA transcript. Thus, *SMN2* produces less full-length mRNA and protein than *SMN1*. We have screened a library of compounds in order to identify ones that can alter the splicing pattern of the *SMN2* gene. Here, we report that the compound aclarubicin increases the retention of exon 7 into the *SMN2* transcript. We show that aclarubicin effectively induces incorporation of exon 7 into *SMN2* transcripts from the endogenous gene in type I SMA fibroblasts as well as into transcripts from a *SMN2* minigene in the motor neuron cell line NSC34. In type I fibroblasts, treatment resulted in an increase in SMN protein and gems to normal levels. Our results suggest that alteration of splicing pattern represents a new approach to modification of gene expression in disease treatment and demonstrate the feasibility of high throughput screens to detect compounds that affect the splicing pattern of a gene.

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a common autosomal recessive disorder characterized by loss of α-motor neurons in the spinal cord (1). SMA is the leading hereditary cause of infant mortality (2). It has an incidence of one in 10 000 live births and a carrier frequency of one in 50 (3–5).

SMA is caused by mutation of the telomeric survival motor neuron gene *SMN1* but not its centromeric copy *SMN2* (6–10). The SMN transcript is encoded by both genes and patients do produce low levels of SMN protein from the *SMN2* gene (6,11,12). The *SMN1* and *SMN2* genes differ functionally by one nucleotide that alters the activity of a splicing enhancer in exon 7 (13,14). As a result, the majority of the transcript derived from the *SMN1* gene contains exon 7 whereas the majority of the transcript from the *SMN2* gene lacks exon 7 (6,15,16). There is a tight correlation between clinical severity of SMA, *SMN2* copy number and the SMN protein level (5,8 and references therein, 10–12,17). SMN is expressed in all tissues and localizes to both the cell cytoplasm and nucleus (18,19). In the nucleus it is concentrated in dot-like structures, called gems, which are often associated with coiled bodies (18,19). In cells and tissues from SMA patients the number of gems is reduced with the most severe, type I, patients showing very few or no gems (11,12,20).

Mice possess only one copy of the SMN gene (*Smn*) that is equivalent to human *SMN1* (21,22). A homozygous knockout of *Smn* results in an embryonic lethal phenotype with massive apoptosis (23). This phenotype indicates that SMN is essential for cell survival. This is consistent with the functions so far attributed to SMN in small nuclear ribonuclear protein (SnRNP) biogenesis (24–26), pre-mRNA splicing (27), interactions with transcription factors (28,29) and other proteins such as profilins (30). Complete loss of SMN affects all cell types presumably because it disrupts these functions. Reduction of SMN, on the other hand, as seen in SMA, causes a rather selective degeneration of motor neurons. The precise function affected is not known.

The introduction of the *SMN2* gene into $Smn^{-/-}$ mice overcomes the embryonic lethality. Mice with one or two copies of *SMN2* develop a severe SMA-like phenotype. With increasing copy number the phenotype gets milder. $Smn^{-/-}$ mice with eight copies of *SMN2* have normal SMN protein levels and gem counts and are phenotypically normal (31–33). In both $Smn^{+/-}$ (34) and $Smn^{-/-}$;*SMN2* (32) mice, loss of motor neurons occurs after birth suggesting that increase of the SMN protein

*To whom correspondence should be addressed at: Department of Molecular and Cellular Biochemistry, Room 363 Hamilton Hall, Ohio State University, 1645 Neil Avenue, Columbus, OH 43210, USA. Tel: +1 614 688 4759; Fax: +1 614 292 4118; Email: burghes.1@osu.edu

produced by *SMN2* could correct the clinical phenotype. SMN protein levels could be raised in SMA patients by stimulating expression of the *SMN2* gene and/or increasing the incorporation of exon 7 into the transcripts from the *SMN2* gene. Recent work has shown that overexpression of the Htra2-β1 protein can correct the splicing pattern of transiently transfected *SMN2* minigenes so that they produce more full-length mRNA (35).

In this paper we identify a drug compound, aclarubicin, that stimulates the incorporation of exon 7 into the processed transcripts from the *SMN2* gene. This increases the amount of full-length transcript produced by the *SMN2* gene, which in turn restores SMN protein levels and nuclear gems to cells derived from type I SMA patients. Our results demonstrate the feasibility of identifying by high throughput screens other compounds and/or aclarubicin derivatives that increase full-length mRNA production and SMN protein from the *SMN2* gene. Moreover, our results suggest that compounds that alter specific splicing patterns of genes may represent a new group of molecules for the development of drugs in disease therapy.

RESULTS

Identification of a compound that increases full-length mRNA from the *SMN2* gene

For initial screens we developed the cell line 3061 from the type I SMA fibroblast cell line 3813 using the transforming activity of the simian virus 40 (SV40) T large antigen (36). The cell line carries two copies of *SMN2* and retains the *SMN2* gene isoform splicing pattern of the parental cell line 3813. Analysis of SMN isoform expression after 24 h of drug treatments with compounds from the Microsource + Library was carried out using a semi-quantitative RT–PCR assay that we have described previously (12). A representative experiment is shown in Figure 1A. The only compound that clearly influenced the incorporation of exon 7 by the *SMN2* gene was aclarubicin (aclacinomycin A) (Fig. 1A, lane 7 and B). The compounds were dissolved in DMSO but DMSO alone had a minimal effect on splicing of the *SMN2* gene (Fig. 1A, lane 8). We also studied the effect of aclarubicin treatment on *SMN2* gene expression for extended periods of time. Prolonged treatment with 10 nM aclarubicin for up to 5 days increased the amount of full-length SMN (Fig. 1C). Densitometric analysis of the results shows that after 48 h treatment aclarubicin induces an ~45% increase in the amount of full-length SMN and a 2-fold increase in the ratio of full-length to Δexon 7 isoforms compared to untreated cells. After 96 h of incubation this ratio increases to >4-fold and the amount of full-length to ~65% more than untreated cells (Fig. 1D). It should also be noted that the levels of total SMN transcript to hypoxanthine phosphoribosyl transferase (HPRT) (the internal control) is not altered in the aclarubicin-treated cells indicating that there is not a selective effect on the turnover rate of one SMN RNA species but a true switch in the amounts of full-length to Δexon 7 isoform. The positive compound aclarubicin was re-tested on the primary SMA fibroblast cell line 3813 with identical results (data not shown).

Time and dosage dependence of aclarubicin to restore full-length SMN levels

To evaluate the persistence of the effect of aclarubicin, 3061 cells were treated with 10 nM aclarubicin for 72 h and, after washing, switched to a drug-free medium. Total RNA was isolated at the indicated time points after the medium change and analyzed for SMN isoform expression (Fig. 2A). The effect of aclarubicin is sustained and prolonged in time (Fig. 2B). Forty-eight hours after removal of the drug, cells still show a 60% increase in full-length SMN compared to untreated cells. The effective concentration of aclarubicin depends on the cell density and in our culture conditions (4000 cells/cm^2 at seeding time) 10 nM represents a critical concentration (Fig. 2C and D). Lower concentrations (0.01–1 nM) were not effective and higher concentrations were not tested as they were cytotoxic. Treatment with 10 nM aclarubicin resulted in decreased cell numbers after 72 h of incubation. The remaining cells were viable and morphologically normal. We also investigated whether continuous cell growth in low aclarubicin concentrations could induce *SMN2* to produce higher amounts of full-length SMN. 3061 cells grown for 2 weeks in medium containing 1 nM aclarubicin showed no alteration in *SMN2* gene splicing (data not shown). To ensure that the effect of aclarubicin was not due to a stress response, we treated cells with aklavin hydrochloride, a structural analog of aclarubicin that shows a similar toxicity profile. Cells challenged with increasing concentrations of aklavin hydrochloride for 5 days showed no alteration in the splicing pattern of the *SMN2* indicating that the aclarubicin effect is not a non-specific cellular stress response (Fig. 3A). Finally, we wished to determine if aclarubicin globally affects the splicing process. The *Myosin V* gene that expresses multiple isoforms in human fibroblasts was chosen for analysis (37). Cells treated with 10 nM aclarubicin for 5 days did not show any alteration in the *Myosin V* splicing pattern (Fig. 3B). Thus, the aclarubicin effect on *SMN2* splicing does not reflect an effect common to all genes.

Restoration of full-length SMN in the motor neuron-like cell line NSC34

The NSC34 cell line was created by fusion of neuroblastoma cells with mouse primary motor neurons (38). This cell line has a number of motor neuron-like characteristics, including expression of cholineacetyl-transferase and the neurofilament triplet proteins (38). A *SMN2* minigene construct consisting of the *SMN2* promoter driving *SMN2* exon 6–8 genomic sequence, with a β-lactamase reporter gene fused to exon 8, was transfected into NSC34 cells. In this construct, exon 7 has been modified to remove the translational termination signal (39). When exon 7 is included in the transcript the β-lactamase sequence is in-frame and translated whereas if exon 7 is excluded the β-lactamase translational reading frame is disrupted and the reporter is not expressed. The exon 6 sequence was also modified, as described in Materials and Methods, to ensure efficient translation.

Twelve stable cell lines were isolated by fluorescent-activated cell sorting (FACS) and the clone 5.3 was selected for further experiments due to its low β-lactamase background. Selection for a low β-lactamase likely results in a clone where the ratio of Δexon 7 to full-length transcript is not the same as that from

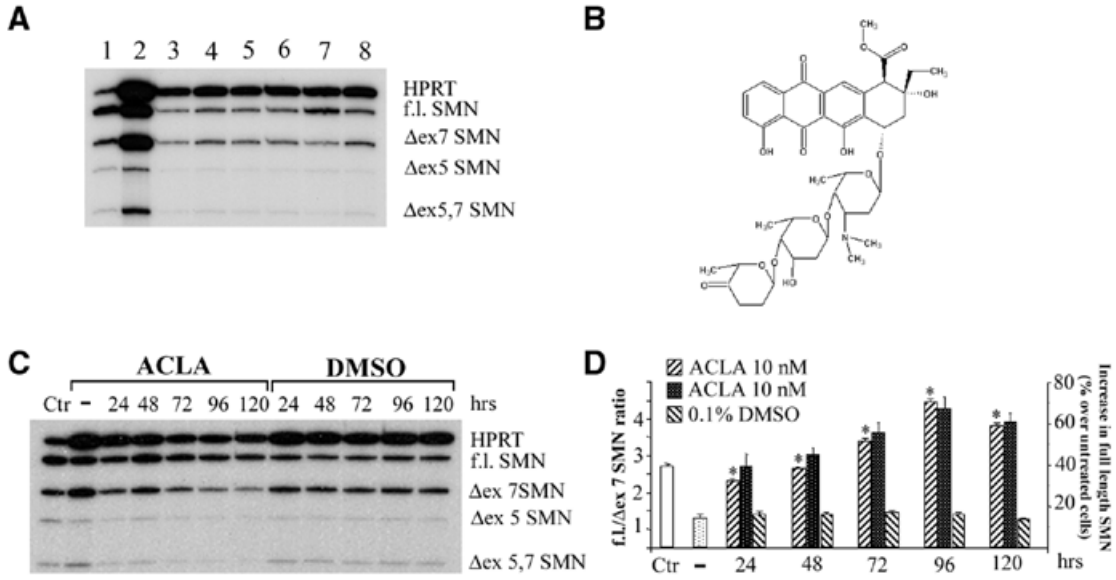

Figure 1. Effect of drug treatment on *SMN2* gene expression in transformed SMA type I fibroblasts 3061. (**A**) Total RNA was extracted from the cells after 24 h of treatment, amplified by RT–PCR and run in denaturing conditions on a 6% polyacrylamide gel. Lane 1, SMA carrier fibroblasts 3814; lanes 2–8, SMA cell line 3061: lane 2, untreated; lane 3, meclocycline treated; lane 4, tetracycline treated; lane 5, methacycline treated; lane 6, doxorubicin treated; lane 7, aclarubicin treated; lane 8, 0.1% DMSO. (**B**) Molecular structure of aclarubicin. (**C** and **D**) Time course of aclarubicin treatment on *SMN2* expression. Fibroblasts cells 3061 were grown for up to 5 days in a medium containing 10 nM aclarubicin or 0.1% DMSO, RNA extracted at the indicated time points (24–120 h) and RT–PCR performed. (C) A representative experiment is shown in the figure. Ctr, untreated SMA carrier fibroblasts 3814; –, untreated SMA I fibroblasts 3061. (D) Semi-quantitative analysis of full-length and Δexon 7 SMN mRNA content of SMA I fibroblasts 3061 treated with 10 nM aclarubicin or 0.1% DMSO for the indicated time period. All readings are the result of at least three independent RT–PCR assays. The white background symbols represent the full-length/Δexon 7 SMN ratio, as indicated on the left *y* axis. Black background symbols represent SMN full-length amount in aclarubicin-treated cells, as indicated on the right *y* axis. ACLA, aclarubicin; f.l., full length; $*P < 0.05$ versus untreated cells.

the endogenous gene. This clone expresses considerably more Δexon 7 transcript. In our experience, the RT–PCR assay used here, with low cycle number and resolution of the PCR products under denaturing conditions, accurately reflects the isoform ratio whereas ethidium bromide stained native gels do not allow a correct evaluation of the relative abundance of the SMN isoforms, mostly because of heteroduplex formation. To evaluate the effect of increasing concentrations of the drug, 5.3 cells were treated with 1–10 nM aclarubicin for 5 days. Inclusion of exon 7 was dosage dependent, with aclarubicin concentrations higher than 4 nM showing a significant increase (Fig. 4A–C). Concentrations higher than 10 nM were toxic. As expected, the increased incorporation of exon 7 into the *SMN2* minigene transcripts was paralleled by an increase in β-lactamase activity (Fig. 4D). The increase in the reporter activity was also confirmed by comparison of DMSO- (Fig. 4E) and aclarubicin-treated (Fig. 4F) cells by fluorescent microscopy analysis.

Restoration of SMN protein levels in cells from type I SMA patients by aclarubicin treatment

Gem number is severely reduced in cells from type I patients (fibroblast cell line 3813: 3.3% of nuclei having gems) compared to cells from a carrier parent (fibroblast cell line 3814: 24.8% of nuclei having gems). SMN protein expression studies were performed on the type I cell line 3813 and compared to the cell line 3814. As the amount of full-length SMN mRNA is increased by aclarubicin treatment, we wished to determine whether there was a concomitant increase in gem numbers. A 5 day incubation of the 3813 cell line with 10 nM aclarubicin increased gem number significantly (Fig. 5A). We observed 22–25% of nuclei having gems in 3813 cells after incubation with aclarubicin. This compares with untreated 3813 cells that have 4–5% of nuclei with gems (Fig. 5B). We have previously shown that primary fibroblasts from type I patients have severely reduced SMN levels (12). Treatment of primary SMA type I fibroblasts 3813 with 10 nM aclarubicin for 5 days increased SMN protein restoring the level to those seen in the fibroblast cell line from the carrier parent 3814 (Fig. 5C).

DISCUSSION

SMA is caused by mutation of the *SMN1* gene with retention of the *SMN2* gene (6–10). The *SMN2* gene is unable to produce sufficient amounts of SMN protein for survival of motor neurons. This is because most of the transcripts from the *SMN2* gene lack exon 7 resulting in a protein which is defective in self oligomerization (40), rapidly degraded and therefore unable to form gems efficiently (41,42). The level of SMN protein correlates with phenotypic severity, with type I SMA patients having the lowest SMN levels and the lowest number of gems (11,12,20). Mice lacking mouse *Smn* and carrying one to two copies of the human *SMN2* gene develop SMA, lack gems and have low SMN levels whereas $Smn^{-/-}$ mice with eight copies of *SMN2* have gems, high SMN levels and a normal phenotype

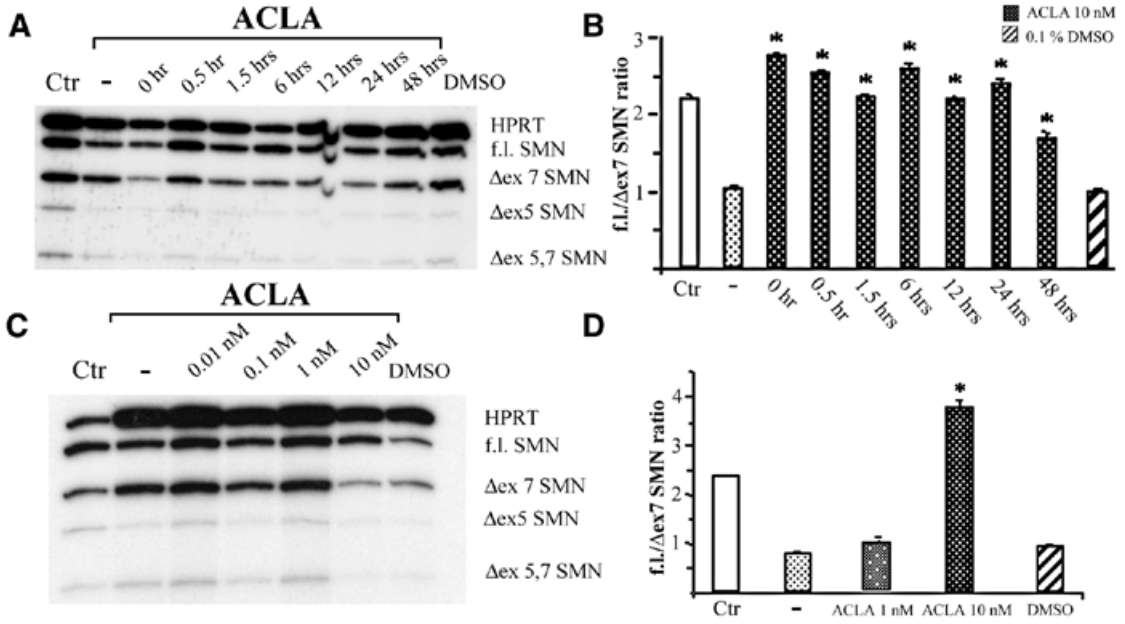

Figure 2. Dosage and time dependence of aclarubicin action. (**A** and **B**) The SMA I cell line 3061 was treated with 10 nM aclarubicin for 72 h, then washed and switched to a drug free medium. Total RNA was isolated at the indicated time points (0–48 h) after removal of aclarubicin and amplified by RT–PCR. (A) The picture represents a typical result of RT–PCR. Ctr, untreated SMA carrier fibroblasts 3814; ACLA, untreated (–) or aclarubicin-treated (0–48 h) SMA I fibroblasts 3061; DMSO, 0.1% DMSO-treated 3061 cells. (B) The ratio of full-length SMN to Δexon 7 was determined by densitometric analysis. All readings are the result of at least three independent RT–PCR assays. (**C** and **D**) Cells were grown for 5 days in a medium containing various amounts (0.01–10 nM) of aclarubicin or 0.1% DMSO. Total RNA was isolated and amplified by RT–PCR. (C) A typical result is shown in the picture. Ctr, untreated SMA carrier fibroblasts 3814; ACLA, untreated (–) or aclarubicin-treated SMA I fibroblasts 3061; DMSO, 3061 cells treated with 0.1% DMSO. (D) Quantification of full-length SMN to Δexon 7 ratio by densitometric scanning. All readings are the result of at least three independent RT–PCR assays. f.l., full length; $*P < 0.05$ versus untreated cells.

(31,32). Thus, increased SMN protein production from the *SMN2* gene early in development can modify or prevent the SMA phenotype. In this paper, we demonstrate that the splicing pattern of the *SMN2* gene can be pharmacologically modified and that increased incorporation of exon 7 into the transcripts from this gene restores protein levels and gem number to normal levels.

Increased SMN production can be obtained by at least three mechanisms: stimulation of the gene promoter, stimulation of the incorporation of exon 7 into the SMN transcripts or stabilization of the SMN protein. Previously an interferon-stimulated response element (ISRE) was identified in the promoters of the *SMN1* and *SMN2* genes (43). Treatment of fibroblasts from type I SMA patients with interferon β and γ was shown to partially restore SMN protein levels, presumably by activating the ISRE (43). SMN promoter activation will increase both full-length SMN and the transcript lacking exon 7. Although high levels of Δexon 7 SMN are unlikely to be toxic, as mice with eight copies of *SMN2* are normal, the exact role and effect of SMN lacking exon 7 is currently unclear. Moreover, it is uncertain if a molecule that could stimulate the *SMN2* promoter sufficiently can be found. The identification of a compound that increases the incorporation of exon 7 into *SMN2* transcripts represents an alternative strategy. These two approaches could also be used together to ensure sufficient SMN levels are reached.

Overexpression of Htra2-β1, a serine/arginine (SR)-like splicing factor (44), has also been shown to modify the splicing pattern of a *SMN2* minigene in transient transfections (35). However, it is difficult to obtain stable transfectants expressing the SR splicing factors (C.Andreassi and A.H.M.Burghes, unpublished data) to demonstrate activity on the endogenous SMN gene.

In the current paper, we demonstrate that aclarubicin acts on the endogenous SMN gene to alter incorporation of exon 7 into the *SMN2* transcripts. Being a drug compound we feel aclarubicin also has the added advantage of being easily deliverable compared to a protein such as Htra2-β1. An intriguing question is the mode of action of aclarubicin. Anthracycline antibiotics such as aclarubicin and doxorubicin are widely used in conventional chemotherapy against solid tumors and leukemias. Anthracyclines have also been shown to be potent differentiation inducers when used at subtoxic concentrations (20–40 nM) (45). Despite the fact that aclarubicin and doxorubicin share closely related structures, their differentiating activities involve distinct regulatory pathways as aclarubicin seems to specifically increase the expression of transcription factors whereas doxorubicin appears to act at the post-transcriptional level by increasing the half-lives of mRNAs (46). It is noteworthy that neither doxorubicin (tested at 10 and 100 nM concentration) nor any other tetracycline derivatives tested show any activity on the *SMN2* gene splicing. This could indicate that the mechanism of aclarubicin action in altering splicing is distinct from other activities of the drug and raises the hope that compounds with less toxic side effects can be found. Aclarubicin has also been shown to

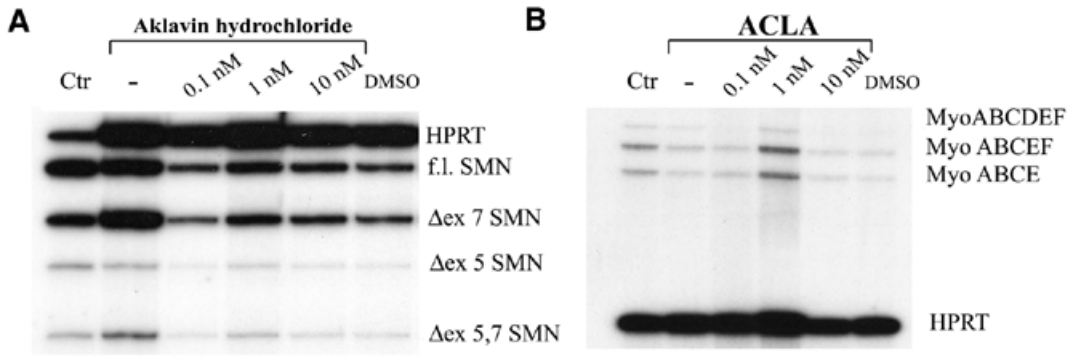

Figure 3. Effect of aklavin hydrochloride on splicing of *SMN2* and effect of aclarubicin on splicing of *Myosin V* gene. (**A**) The SMA I cell line 3061 was treated with increasing concentrations (0.1–10 nM) of aklavin hydrochloride for 5 days. The medium was changed daily and fresh compound added. Ctr, untreated SMA carrier fibroblasts 3814; Aklavin hydrochloride, untreated (–) or aklavin hydrochloride (0.1–10 nM)-treated SMA I fibroblasts 3061; DMSO, 3061 cells treated with 0.1% DMSO. Notice that despite a similar toxicity profile to aclarubicin there is no effect on *SMN2* splicing. (**B**) The SMA cell line 3061 was treated with increasing concentrations of aclarubicin and RNA isolated and analyzed by RT–PCR for the *Myosin V* gene expression pattern. Ctr, untreated SMA carrier fibroblasts 3814; ACLA, untreated (–) or aclarubicin (0.1–10 nM)-treated SMA I fibroblasts 3061; DMSO, 3061 cells treated with 0.1% DMSO. f.l., full length.

interact with the nuclear enzyme type II DNA topoisomerase (47). This enzyme, besides its recognized activity in DNA metabolism, has been shown to play a critical role in motor neuron development as mice lacking the murine DNA topoisomerase type IIβ express a defect in motor neuron axon growth and die shortly after birth (48). Experiments using drugs known to inhibit topoisomerase II or to mimic other identified actions of aclarubicin along with *in vitro* splicing assays will be required to dissect the mechanism of aclarubicin activity. Our data indicate that treatment of cells with aclarubicin does not alter the splicing of all genes as the *Myosin V* gene showed no modification in the spliced transcripts produced. SR-rich proteins play an important role in constitutive and alternative pre-mRNA splicing. The activity and the cellular localization of these proteins are regulated by phosphorylation (49). Immunofluorescence experiments show that aclarubicin treatment induces a redistribution of SR proteins (C.Andreassi and A.H.M.Burghes, unpublished data) suggesting a modification in the status of these proteins. Analysis of the splicing pattern of a larger number of genes, in particular those containing a SR protein recognition sequence similar to that of *SMN2*, is required to determine the specificity of aclarubicin action. This underscores the importance of understanding which SR proteins bind to the splice enhancer in *SMN* exon 7. Aclarubicin is also active in a β-lactamase reporter gene assay designed to detect increased exon 7 incorporation into the transcripts from a *SMN2* minigene construct. The results using the motor neuron cell line NSC34 indicate that aclarubicin activity is conserved across species and in different cell types. This cell line and assay system is amenable to high throughput screens for the identification of additional compounds able to increase the production of full-length SMN from *SMN2*.

Although aclarubicin does restore SMN levels in cells from type I patients, it is most commonly used as a chemotherapeutic agent for treating cancer. Thus, its side effects and known toxicity make it unsuitable for consideration in the treatment of young SMA patients (50). Very recently, Chang *et al.* (51) reported that sodium butyrate treatment of SMA lymphoid cell lines alters the splicing pattern of the *SMN2* gene. Using the same semi-quantitative assay described here, we observed that a 24 h treatment of SMA I fibroblast cell line 3061 with 500 ng/ml sodium butyrate induces a 2.3-fold increase in full-length/Δexon 7 SMN ratio (data not shown) compared to 1.8-fold with 10 nM aclarubicin. In addition, sodium butyrate elevated expression levels of both the internal control HPRT and SMN. This effect is probably due to sodium butyrate's known activity as a histone deacetylase inhibitor and thus to non-specific transcriptional activation. In our hands aclarubicin does not activate *SMN* or *HPRT* gene expression. Sodium butyrate treatment was also tested in a mouse model of SMA (51) but it remains unclear whether sufficient levels of the drug were reached *in vivo* to alter the splicing pattern of the gene in all tissues. Moreover, treatment of pregnant animals still resulted in a large number of SMA offspring. Thus, there is the need for identification of a panel of compounds whose efficacy *in vivo* has to be tested in animal models of SMA. The identification of highly active compounds can be achieved by the high throughput screens outlined in this report.

Our data demonstrate that alteration of splicing patterns can represent a novel target for altering gene expression. As compounds like aclarubicin that increase exon incorporation can be identified, it is presumed that compounds that decrease the incorporation of an exon (exon skipping) into transcripts can be also found. Alteration of gene expression pattern is a common phenomenon associated with cancer (52) or the result of mutations in genetic disease (53). For example, nonsense mutations in exon 18 of the breast cancer susceptibility gene *BRCA1* cause skipping of this exon with subsequent disruption of the first BRCA1 protein domain (54). Some missense and silent mutations also result in exon skipping, for instance, the silent mutations in *Tau* which cause frontotemporal dementia (55). Therefore, an understanding of how drug compounds can alter the splicing of *SMN2* will not only be useful for the development of treatments for SMA but may also give insight into the development of therapies for other disorders. In muscular dystrophy, for instance, compounds that enhance

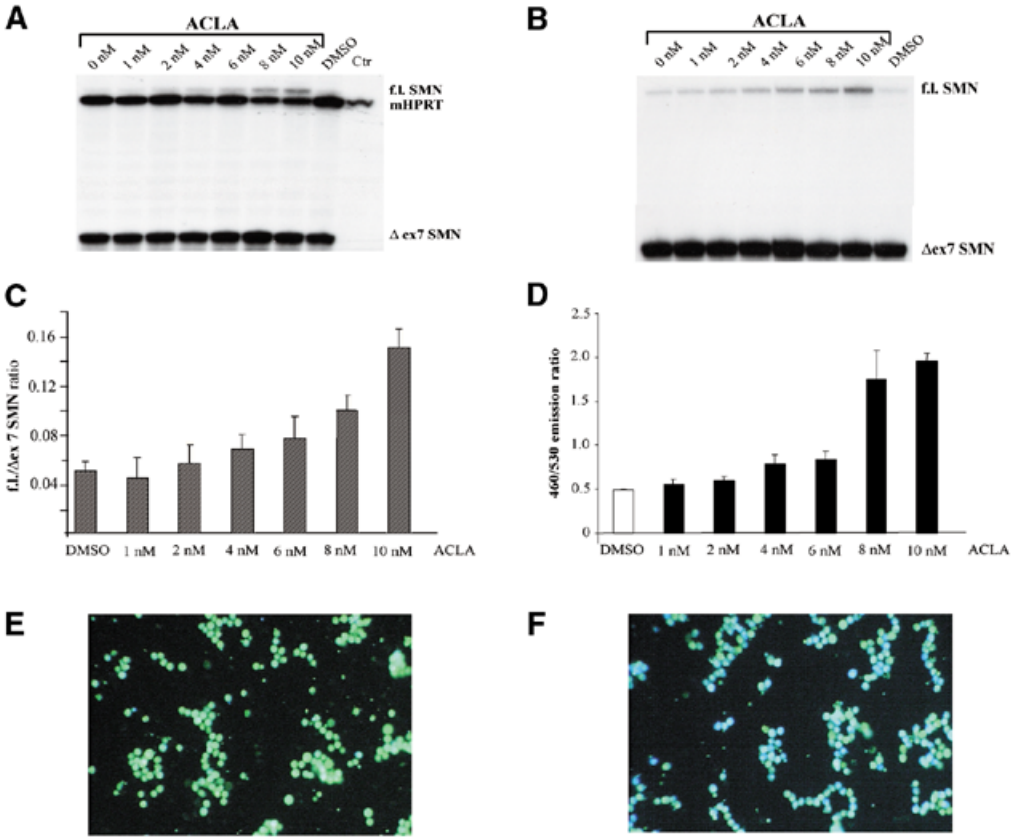

Figure 4. Aclarubicin treatment of NSC34 containing a *SMN2* minigene reporter. The cell line 5.3 was derived from the motor neuron cell line NSC34 by transfection with a *SMN2* mingene reporter. In these cells, when exon 7 is incorporated into the *SMN2* minigene transcript the β-lactamase is produced. (**A** and **B**) The 5.3 cells were grown for 5 days in a medium containing various amounts (1–10 nM) of aclarubicin or 0.1% DMSO. RNA was isolated from the cells and RT–PCR performed using primers that specifically amplified the minigene products. (A) A representative RT–PCR result is shown. (B) RT–PCR from the same samples was performed without the control HPRT primers. ACLA, untreated (0 nM) or aclarubicin (1–10 nM)-treated 5.3 cells; DMSO, 5.3 cells treated with DMSO; Ctr, NSC34 parental cells. (**C**) The ratio of full-length to Δexon 7 SMN in 5.3-treated cells was determined by densitometric scanning. All readings are the result of at least three independent RT–PCR assays. (**D**) β-Lactamase activity of aclarubicin- or DMSO-treated cells. The reporter activity is represented as the ratio of substrate emission values at 460 nM (blue) and 530 nM (green), where a higher ratio represents increased β-lactamase activity. (**E**) Fluorescence microscopy analysis of DMSO-treated 5.3 cells. (**F**) Fluroscence microscopy analysis of 5.3 cells treated for 5 days with 10 nM aclarubicin. Note the shift from green cells in (E) to blue cells in (F) indicating increased amounts of β-lactamase in treated cells.

skipping of exons in the *Dystrophin* gene could be useful to restore reading frame to the dystrophin mRNA and allow production of more dystrophin (56). Conversely, compounds that induce exon incorporation could, for instance, be used to stimulate the apoptosis-related *Caspase 2* gene to produce full-length Ich-1L, instead of its inactive splice variant Ich-1S, in order to sensitize cancer cells to chemotherapeutic agents (57).

MATERIALS AND METHODS

Cell culture and treatment

Human SMA type I fibroblasts 3813 (12), SV-40 transformed SMA I fibroblasts 3061 and SMA carrier fibroblasts 3814 (12) were grown in DMEM medium containing 10% (v/v) fetal calf serum, 2 mM glutamine and antibiotics. The mouse motor neuron cell line NSC34 (38) was cultured in DMEM containing 5% (v/v) fetal bovine serum, 2 mM glutamine and antibiotics. In all instances the cells were plated the day preceding treatment with drug compound(s) and harvested prior to reaching confluency. The fibroblast cell line 3061 was seeded at 4000 cells/cm^2 and the NSC34 clone 5.3 at 6000 cells/cm^2. The compounds were obtained from Microsource Discovery Systems (www.msdiscovery.com) as part of the Genesis Plus collection. The drugs were dissolved in DMSO in microtiter plates and used at a 1/1000 dilution (the complete list of tested compounds is available upon request). In prolonged treatment experiments the medium was changed daily and fresh compound was added. For subsequent tests aclarubicin was obtained from either Sigma (90% pure) or Affinity (95% pure).

Constructs and transfections

SV40 viral DNA (Gibco BRL) was digested with *Bam*HI and subcloned into the pcDNA$_3$ vector (41). The resulting clone was digested with *Xba*I and purified DNA was used to transfect 5×10^4 3813 cells using the Effectene reagent (Gibco

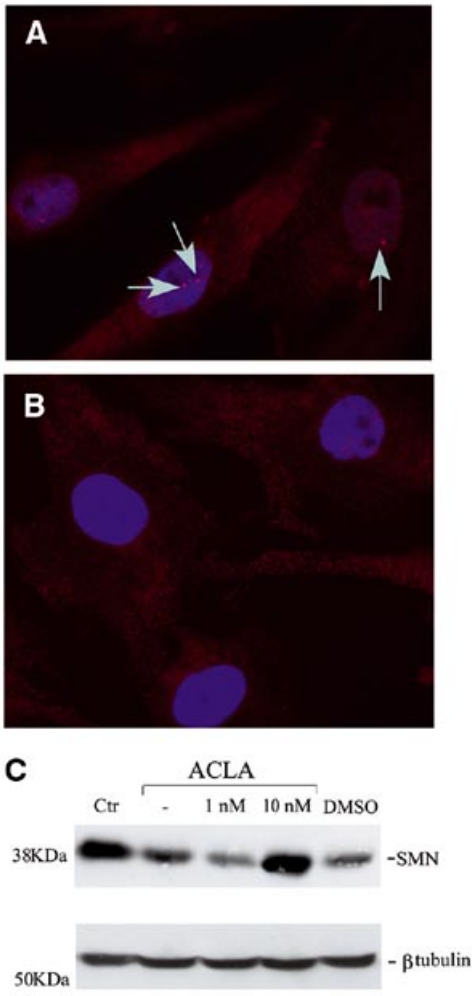

Figure 5. SMN protein expression in aclarubicin-treated SMA I fibroblasts. 3813 cells were grown for 5 days in the presence (**A**) or absence (**B**) of 10 nM aclarubicin and then stained for SMN. Anti-SMN-specific antibody MANSMA2 was used to detect SMN protein. The cell nucleus is counterstained by the nuclear dye DAPI. Treated cells show 27–34% of gem positive nuclei compared to 8–10% gem positive nuclei in untreated cells. Magnification 1000×. (**C**) 3813 cells were grown for 5 days in the absence or presence of 1 or 10 nM aclarubicin. As controls, carrier fibroblasts 3814- and 0.1% DMSO-treated cells are included. Total protein was solubilized and run on a 12% polyacrylamide gel and transferred to a filter. The filter was probed with anti-SMN antibody MANSMA2 to reveal a 38 kDa band corresponding to SMN. The same blot after stripping off the antibody was reprobed with anti-β-tubulin antibody (50 kDa) to control for protein amounts in individual lanes. As indicated by the western blot results, total SMN levels increase, thus, cytoplasmic SMN staining is also enhanced in aclarubicin-treated cells.

BRL). Stable clones were selected using G418 at 500 µg/ml for 8–10 days. Cloned colonies were screened by PCR for the presence of SV40 and growth of the colonies monitored. For the work described here clone 3061 was selected.

The minigene containing *SMN2* exon 6–8 genomic sequence has been described previously (14). It was used as a template to generate a β-lactamase reporter construct using the same strategy described elsewhere (39). The primers for β-lactamase amplification were Ex8-lac (GAAATGCTGGCATAGAGCAGCTGGACCCAGAAACGCTGGTGAAAG) and lac-stop (TAGCGGCCGCTAGATTACCAATGCTTAATC). A 7.2 kb fragment containing the minigene β-lactamase fused sequence was released using *Xho*I and *Not*I, the sites end filled, and the fragment subcloned into a blunt ended *Kpn*I site of pSMN2Script (pCMVScript with the CMV promoter replaced by the 3.4 kb *SMN2* promoter) (58). The *SMN2* exon 6 sequence was modified to include an in-frame ATG and a Kozak sequence (CCCATATGT altered to ACCATGTGG). The minigene plasmid was amplified using the 5′ CACACACACACTGGAGTTC primer and the 3′ ATCAAGAGAATCTGGCCACATGGTAGGTGGTGGGGGAAT primer. The 0.5 kb product together with a 3′ primer GCCTATCTCAACCACGTGGC was used to amplify the minigene plasmids so as to obtain a 3.8 kb product containing the modified sequence. The PCR product was first cloned into a TOPO TA vector (Invitrogen), the insert released with *Apa*I and *Bbr*P1 and subcloned into pSMN2Script. The modified sequence was verified by sequencing. 5×10^5 NSC34 cells were transfected with modified *SMN2* minigene plasmid using Lipofectamine reagent (Gibco BRL). Stable clones were selected with 500 µg/ml G418 for 12 days. To isolate single cell colonies, the *SMN2* minigene selected cells were loaded with a fluorescent substrate of β-lactamase, CCF2/AM (Aurora Biosciences) and FACS sorted as described by Zlokarnik *et al.* (59). Clonal cell lines expressing low basal levels of β-lactamase were expanded for 3 weeks. Clone 5.3 was selected for the experiments described here. β-Lactamase activity was assessed by the emission shift of CCF2/AM as described previously (59).

Semi-quantitative RT–PCR analysis of SMN transcripts

Total RNA was isolated from untreated or treated fibroblast cells using the TRizol reagent (Gibco BRL) and first-strand cDNA synthesis made as described previously (9). To amplify the endogenous SMN transcripts, a multiplex RT–PCR was performed using primers and conditions described previously (9). This reaction yields four possible SMN RT–PCR products (full-length SMN transcript and isoforms lacking exon 5 and/or 7) and one HPRT RT–PCR product as a loading control for the amounts of mRNA. To amplify transcripts from the *SMN2* minigene β-lactamase construct, a multiplex RT–PCR was performed using the following primers SMN6 forward (CACCTACCATGTGGCCAGATTC) and the SMN8lact reverse (TTCACCCGAGTTTCTGGGGTCCAGC) using conditions described previously (9), giving two possible PCR products (+SMN exon 7 and –SMN exon 7). The transcript from the mouse HPRT gene was coamplified using the mHPRT forward (GGTTAAGGTTGCAAGCTTCGTGG) and the mHPRT reverse (GGCAACATCAAGCTTGCTGG) primers. This allows control for equal amounts of template. The resulting PCR products were electrophoresed on a 6% denaturing polyacrylamide gel and the dried gel either exposed to Hyperfilm (Amersham) for different times or to a Storm Imager cassette. Analysis was performed either using the Storm Imager Software (Amersham) or by densitometry of the bands on a Shimadzu CS-9000 densitometer. The amounts of the SMN isoforms and the HPRT transcripts were determined for all samples (5,9). Statistical analysis of the data was performed using one-way ANOVA and a *P*-value of at least 0.05 was considered significant. The human *Myosin V* gene

transcripts were analyzed using primers and conditions described previously (37) except that primers were end labeled and the PCR products run on a denaturing gel, as described above.

Immunocytochemistry

Immunofluorescence staining of fibroblast cells was performed as described previously (12,41). The MANSMA2 anti-SMN monoclonal antibody (19) diluted 1:100 was used to detect gems as described previously (12,41). Anti-SMN antibody binding was visualized using a biotin-conjugated anti-mouse secondary antibody and subsequently Cy3-conjugated streptavidin. Cell staining was visualized using a Nikon microscope equipped with a dual band pass DAPI/FITC filter and images were captured with a Magnafire digital camera (Optronics).

Western blot analysis

Preparation of the sample, electrophoresis and protein blotting were performed as described previously (12). The MANSMA2 anti-SMN monoclonal antibody diluted 1:1000 was used to detect SMN and the ECL system (Amersham) used for detection. Filters were stripped and reprobed with an anti-β-tubulin antibody (Sigma) to control for loading.

ACKNOWLEDGEMENTS

We thank Dr Neil Cashman for kindly providing the NSC34 cells and Kristie Schussler for technical assistance. C.A. is the recipient of a Post-doctoral Fellowship from Families of SMA. Funding for these studies was supported by Families of SMA, the Madison, Mathew and Preston funds and NIH grant NS38650 to A.H.M.B., NIH grant NS40275 to E.A. J.Z. is supported by Andrew's Buddies.

REFERENCES

1. Crawford, T.O. and Pardo, C.A. (1996) The neurobiology of childhood spinal muscular atrophy. *Neurobiol. Dis.*, **3**, 97–110.
2. Roberts, D.F., Chavez, J. and Court, S.D.M. (1970) The genetic component in child mortality. *Arch. Dis. Child.*, **45**, 33–38.
3. Pearn, J. (1973) The gene frequency of acute Werdnig-Hoffmann disease (SMA type 1). A total population survey in North-East England. *J. Med. Genet.*, **10**, 260–265.
4. Pearn, J. (1978) Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. *J. Med. Genet.*, **15**, 409–413.
5. McAndrew, P.E., Parsons, D.W., Simard, L.R., Rochette, C., Ray, P., Mendell, J.R., Prior, T.W. and Burghes, A.H.M. (1997) Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *Am. J. Hum. Genet.*, **60**, 1411–1422.
6. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millaseau, P., Zeviani, M. *et al.* (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, **80**, 155–165.
7. Hahnen, E., Forkert, R., Merke, C., RudnikSchoneborn, S., Schonling, J., Zerres, K. and Wirth, B. (1995) Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homozygous deletions of the SMN gene in unaffected individuals. *Hum. Mol. Genet.*, **4**, 1927–1933.
8. Burghes, A.H.M. (1997) When is a deletion not a deletion? When it is converted. *Am. J. Hum. Genet.*, **61**, 9–15.
9. Parsons, D.W., McAndrew, P.E., Iannaccone, S.T., Mendell, J.R., Burghes, A.H.M. and Prior, T.W. (1998) Intragenic telSMN mutations: frequency, distribution, evidence of a founder effect, and modification of the spinal muscular atrophy phenotype by cenSMN copy number. *Am. J. Hum. Genet.*, **63**, 1712–1723.
10. Wirth, B., Herz, M., Wetter, A., Moskau, S., Hahnen, E., Rudnik-Schoneborn, S. and Zerres, K. (1999) Quantitative analysis of survival motor neuron copies: identification of subtle *SMN1* mutations in patients with spinal muscular atrophy, genotype–phenotype correlation, and implications for genetic counseling. *Am. J. Hum. Genet.*, **64**, 1340–1356.
11. Lefebvre, S., Burlet, P., Liu, Q., Bertrandy, S., Clermont, O., Munnich, A., Dreyfuss, G. and Melki, J. (1997) Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.*, **16**, 265–269.
12. Coovert, D., Le, T.T., McAndrew, P., Strasswimmer, J., Crawford, T.O., Mendell, J.R., Coulson, S., Androphy, E.J., Prior, T.W. and Burghes, A.H.M. (1997) The survival motor neuron protein in spinal muscular atrophy. *Hum. Mol. Genet.*, **6**, 1205–1214.
13. Monani, U.R., Lorson, C.L., Parsons, D.W., Prior, T.W., Androphy, E.J., Burghes, A.H.M. and McPherson, J.D. (1999) A single nucleotide difference that alters splicing patterns distinguishes the SMA gene *SMN1* from the copy gene *SMN2*. *Hum. Mol. Genet.*, **8**, 1177–1183.
14. Lorson, C.L., Hahnen, E.J., Androphy, E. and Wirth, B. (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl Acad. Sci. USA*, **96**, 6307–6311.
15. Gennarelli, M., Lucarelli, M., Capon, F., Pizzuiti, A., Merlini., L., Angelini, C., Novelli, G. and Dallapiccola, B. (1995) Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. *Biochem. Biophys. Res. Commun.*, **213**, 342–348.
16. Parsons, D.W., McAndrew, P.E., Monani, U.R., Mendell, J.R., Burghes, A.H.M. and Prior, T.W. (1996) An 11 base pair duplication in exon 6 of the SMN gene produces a type I spinal muscular atrophy (SMA) phenotype: further evidence for SMN as the primary SMA-determining gene. *Hum. Mol. Genet.*, **5**, 1727–1732.
17. Vitali, T., Sossi, V., Tiziano, F., Zappata, S., Giuli, A., Paravatou-Petsotas, M., Neri, G. and Brahe, C. (1999) Detection of the survival motor neuron (SMN) genes by FISH: further evidence for a role for *SMN2* in the modulation of disease severity in SMA patients. *Hum. Mol. Genet.*, **8**, 2525–2532.
18. Liu, Q. and Dreyfuss, G. (1996) A novel nuclear structure containing the survival of motor neurons protein. *EMBO J.*, **15**, 3555–3564.
19. Young, P.J., Le, T.T., thi Man, N., Burghes, A.H.M. and Morris, G.E. (2000) The relationship between SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in differentiated tissues and cultured cells. *Exp. Cell Res.*, **25**, 365–374.
20. Patrizi, A.L., Tiziano, F., Zappata, S., Donati, M.A., Neri, G. and Brahe, C. (1999) SMN protein analysis in fibroblast, amniocyte and CVS cultures from spinal muscular atrophy patients and its relevance for diagnosis. *Eur. J. Hum. Genet.*, **7**, 301–309.
21. DiDonato, C.J., Chen, X., Noya, D., Kroenberg, J.R., Nadeau, J. and Simard, L.R. (1997) Cloning, characterization, and copy number of the murine survival motor neuron gene: homolog of the spinal muscular atrophy-determining gene. *Genome Res.*, **7**, 339–351.
22. Viollet, L., Bertrandy, S., Brunialti, A.L., Lefebvre, S., Burlet, P., Clermont, O., Cruaud, C., Guenet, J.L., Munnuch, A. and Melki, J. (1997) cDNA isolation, expression, and chromosomal localization of the mouse survival motor neuron gene (Smn). *Genomics*, **40**, 185–188.
23. Schrank, B., Gotz, R., Gunnerson, J.M., Ure, J.M., Toyka, K., Smith, A. and Sendtner, M. (1997) Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc. Natl Acad. Sci. USA*, **94**, 9920–9925.
24. Liu, Q., Fischer, U., Wang, F. and Dreyfuss, G. (1997) The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell*, **90**, 1013–1022.
25. Fischer, U., Liu, Q. and Dreyfuss, G. (1997) The SMN–SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell*, **90**, 1023–1029.
26. Buhler, D., Raker, V., Luhrmann, R. and Fischer, U. (1999) Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. *Hum. Mol. Genet.*, **13**, 2351–2357.
27. Pellizzoni, L., Katoaka, N., Charroux, B. and Dreyfuss, G. (1998) A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell*, **95**, 615–624.
28. Strasswimmer, J., Lorson, C.L., Breiding, D.E., Chen, J.J., Le, T., Burghes A.H.M. and Androphy, E.J. (1999) Identification of survival motor neuron as a transcriptional activator-binding protein. *Hum. Mol. Genet.*, **8**, 1219–1226.

29. Williams, B.Y., Hamilton, S.L. and Sarkar, H.K. (2000) The survival motor neuron protein interacts with the transactivator FUSE binding protein from human fetal brain. *FEBS Lett.*, **470**, 207–210.
30. Giesemann, T., Rathke-Hartlieb, S., Rothkegel, M., Bartsch, J.W., Buchmeier, S., Jockusch, B.M. and Jockusch, H. (1999) A role for polyproline motifs in the spinal muscular atrophy protein SMN. Profilins bind to and colocalize with smn in nuclear gems. *J. Biol. Chem.*, **274**, 37908–37914.
31. Hsieh-Li, H., Chang, J.G., Jong, Y.J., Wu., M.H., Wang, N., Tsai, C.H. and Li, H. (2000) A mouse model for spinal muscular atrophy. *Nat. Genet.*, **24**, 66–70.
32. Monani, U.R., Sendtner, M., Coovert, D.D., Parsons, D.W., Andreassi, C., Le, T.T., Jablonka, S., Schrank, B., Rossol, W., Prior, T.W. *et al.* (2000) The human centromeric survival motor neuron gene (*SMN2*) rescues embryonic lethality in Smn(–/–) mice and results in a mouse with spinal muscular atrophy. *Hum. Mol. Genet.*, **9**, 333–339.
33. Monani, U.R., Coovert, D.D. and Burghes, A.H.M. (2000) Animal models of spinal muscular atrophy. *Hum. Mol. Genet.*, **9**, 2451–2457.
34. Jablonka, S., Schrank, B., Kralewski, M., Rossoll, W. and Sendtner, M. (2000) Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III. *Hum. Mol. Genet.*, **9**, 341–346.
35. Hofmann, Y., Lorson, C.L., Stamm, S., Androphy, E.J. and Wirth, B. (2000) Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III. *Proc. Natl Acad. Sci. USA*, **97**, 9618–9623.
36. Ozer, H.L., Banga, S.S., Dasgupta, T., Houghton, J., Hubbard, K., Jha, K.K., Kim, S.H., Lenahan, M., Pang, Z., Pardinas, J.R. and Patsalis, P.C. (1996) SV40-mediated immortalization of human fibroblasts. *Exp. Gerontol.*, **31**, 303–310.
37. Lambert, J., Naeyaert, J.M., Callens, T., Paepe, A.D. and Messiaen, L. (1998) Human myosin V gene produces different transcripts in a cell type-specific manner. *Biochem. Biophys. Res. Commun.*, **252**, 329–333.
38. Cashman, N.R., Durham, H.D., Blusztajn, J.K., Oda, K., Tabira, T., Shaw, I.L., Dahrouge, S. and Antel, J.P. (1992) Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons. *Dev. Dyn.*, **194**, 209–21.
39. Zhang, M., Lorson, C.L., Androphy, E.J. and Zhou, J.(2001) An *in vivo* reporter system for measuring increased inclusion of exon 7 in SMN2 mRNA: potential therapy of SMA. *Gene Ther.*, **20**, 1532–1538.
40. Lorson, C.L., Strasswimmer, J., Yao, J.M., Baleja, J.D., Hahnen, E., Wirth, B., Le, T., Burghes, A.H.M. and Androphy, E. (1998) SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nat. Genet.*, **19**, 63–66.
41. Le, T.T., Coovert, D.D., Monani, U.R., Morris, G.E. and Burghes, A.H.M. (2000) The survival motor neuron (SMN) protein: effect of exon loss and mutation on protein localization. *Neurogenetics*, **3**, 7–16.
42. Lorson, C.L. and Androphy, E.J. (2000) An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum. Mol. Genet.*, **9**, 259–265.
43. Baron-Delage, S., Abadie, A., Echaniz-Laguna, A., Melki, J. and Beretta, L. (2000) Interferons and IRF-1 induce expression of the survival motor neuron (SMN) genes. *Mol. Med.*, **6**, 957–968.
44. Caceres, J.F. and Krainer, A.R. (1997) In Krainer, A.R. (ed.), *Eukaryotic mRNA Processing*. IRL Press, Oxford, UK, pp. 174–212.
45. Chénais, B., Andriollo, M., Guiraud, P., Belhoussine, R. and Jeannesson, P. (2000) Oxidative stress involvement in chemically induced differentiation of K562 cells. *Free Radic. Biol. Med.*, **28**, 18–27.
46. Morceau, F., Chenais, B., Gillet, R., Jardillier, J.C., Jeannesson, P. and Trentesaux, C. (1996) Transcriptional and post-transcriptional regulation of erythroid gene expression in anthracycline-induced differentiation of human erythroleukemic cells. *Cell Growth Differ.*, **7**, 1023–1029.
47. Sehested, M. and Jensen, P.B. (1996) Mapping of DNA topoisomerase II poisons (etoposide, clerocidin) and catalytic inhibitors (aclarubicin, ICRF-187) to four distinct steps in the topoisomerase II catalytic cycle. *Biochem. Pharmacol.*, **51**, 879–886.
48. Yang, X., Li, W., Prescott, E.D., Burden, S.J. and Wang, J.C. (2000) DNA topoisomerase II β and neural development. *Science*, **287**, 131–134.
49. Blencowe, B.J., Bowman, J.A., McCracken, S. and Rosonina, E. (1999) SR-related proteins and the processing of messenger RNA precursors. *Biochem. Cell Biol.*, **77**, 277–291.
50. Van Echo, D.A., Whitacre, M.Y., Aisner, J., Applefeld, M.M. and Wiernik, P.H. (1982) Phase I trial of aclacinomycin A. *Cancer Treat. Rep.*, **66**, 1127–1132.
51. Chang, J.G., Hsieh-Li, H.M., Jong, Y.J., Wang, N.M., Tsai, C.H. and Li, H. (2001) Treatment of spinal muscular atrophy by sodium butyrate. *Proc. Natl Acad. Sci. USA*, **98**, 9808–9813.
52. Mercatante, D. and Kole, R. (2000) Modification of alternative splicing pathways as a potential approach to chemotherapy. *Pharmacol. Ther.*, **85**, 237–243.
53. Cooper, T.A. and Mattox, W. (1997) The regulation of splice-site selection, and its role in human disease. *Am. J. Hum. Genet.*, **61**, 259–266.
54. Liu, H.X., Cartegni, L., Zhang, M.G. and Krainer, A.R. (2001) A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. *Nat. Genet.*, **27**, 55–58.
55. D'Souza, I., Poorkaj, P., Hong, M., Nochlin, D., Lee, V.M., Bird, T.D. and Schellenberg, G.D. (1999) Missense and silent tau gene mutations cause frontotemporal dementia with parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements. *Proc. Natl Acad. Sci. USA*, **96**, 5598–5603.
56. Ginjaar, I.B., Kneppers, A.L., v d Meulen, J.D., Anderson, L.V., Bremmer-Bout, M., van Deutekom, J.C., Weegenaar, J., den Dunnen, J.T. and Bakker, E. (2000) Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. *Eur. J. Hum. Genet.*, **8**, 793–796.
57. Fujimura, M., Kasahara, K., Shirasaki, H., Heki, U., Iwasa, K., Ueda, A. and Matsuda, T.J. (1999) Up-regulation of ICH-1L protein by thromboxane A2 antagonists enhances cisplatin-induced apoptosis in non-small-cell lung-cancer cell lines. *J. Cancer Res. Clin. Oncol.*, **125**, 389–394.
58. Monani, U.R., McPherson, J.D. and Burghes, A.H.M. (1999) Promoter analysis of the human centromeric and telomeric survival motor neuron genes (SMNC and SMNT). *Biochim. Biophys. Acta*, **1445**, 330–336.
59. Zlokarnik, G., Negulescu, P.A., Knapp, T.E., Mere, L., Burres, N., Feng, L., Whitney, M., Roemer, K. and Tsien, R.Y. (1998) Quantitation of transcription and clonal selection of single living cells wih β-lactamase as a reporter. *Science*, **279**, 84–88.