A Functional Interaction between the Survival Motor Neuron Complex and RNA Polymerase II

Livio Pellizzoni,* Bernard Charroux,* Juri Rappsilber,‡ Matthias Mann,‡ and Gideon Dreyfuss*

*Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; and ‡Protein Interaction Laboratory, University of Southern Denmark, DK-5230 Odense M, Denmark

Abstract. The survival motor neuron (SMN) protein, the protein product of the spinal muscular atrophy (SMA) disease gene, plays a role in the assembly and regeneration of small nuclear ribonucleoproteins (snRNPs) and spliceosomes. By nanoelectrospray mass spectrometry, we identified RNA helicase A (RHA) as an SMN complex–associated protein. RHA is a DEAH box RNA helicase which binds RNA polymerase II (pol II) and reportedly functions in transcription. SMN interacts with RHA in vitro, and this interaction is impaired in mutant SMNs found in SMA patients. Coimmunoprecipitation demonstrated that the SMN complex is associated with pol II, snRNPs, and RHA in vivo. In vitro experiments suggest that RHA mediates the association of SMN with the COOH-terminal domain of pol II. Moreover, transfection of cells with a dominant negative mutant of SMN, SMNΔN27, causes accumulation of pol II, snRNPs, and RHA in nuclear structures that contain the known markers of gems and coiled bodies, and inhibits RNA pol I and pol II transcription in vivo. These findings indicate a functional as well as physical association of the SMN complex with pol II and suggest a role for the SMN complex in the assembly of the pol II transcription/processing machinery.

Key words: survival motor neuron • RNA helicase A • RNA polymerase II • nuclear bodies • transcriptosome

Introduction

Spinal muscular atrophy (SMA)[1] is an autosomal recessive disease and the most common genetic cause of infant death (Pearn, 1980). SMA is characterized by degeneration of motor neurons in the anterior horn of the spinal cord, resulting in muscular weakness and atrophy (for review see Melki, 1997). The survival motor neuron (SMN) gene is the SMA determining gene (Lefebvre et al., 1995). In humans, the SMN gene is duplicated as an inverted repeat on chromosome 5, and only deletions or mutations in the telomeric copy, SMN1, result in SMA (for review see Burghes, 1997). The centromeric copy, SMN2, does not protect from SMA because, as a result of a single nucleotide difference from SMN1, it produces mostly a nonfunctional truncated form of SMN lacking the COOH-terminal amino acids encoded by exon 7 (Lefebvre et al., 1995; Lorson et al., 1998, 1999; Pellizzoni et al., 1998, 1999; Charroux et al., 2000). SMN is expressed in all tissues of mammalian organisms, and its function is essential in humans, mice, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* (Schrank et al., 1997; Miguel-Aliaga et al., 1999; Hannus et al., 2000; Owen et al., 2000; Paushkin et al., 2000). The amount of full length SMN produced by SMN2 is sufficient to overcome lethality in most cells but not in motor neurons where the amount of SMN inversely correlates with the severity of the disease (Coovert et al., 1997; Lefebvre et al., 1997; Frugier et al., 2000; Hsieh-Li et al., 2000; Jablonka et al., 2000; Monani et al., 2000). Therefore, motor neurons appear significantly more sensitive to SMN reduction than other cell types.

SMN localizes both in the cytoplasm and in gems, nuclear bodies similar in size and number to coiled bodies and often associated with them (Liu and Dreyfuss, 1996). Recently, it has been suggested that coiled bodies be renamed as Cajal bodies (Gall et al., 1999). SMN is tightly associated with Gemin2 (formerly SIP1), Gemin3, and Gemin4 as part of a large multiprotein complex (Liu et al., 1997; Charroux et al., 1999, 2000). In the cytoplasm, the SMN complex is also associated with the Smith antigen (Sm) core proteins of snRNPs, suggesting a function in some aspects of snRNP metabolism (Liu et al., 1997; Charroux et al., 1999, 2000).

Address correspondence to Gideon Dreyfuss, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, 415 Curie Blvd., Clinical Research Bldg., Rm. 328, Philadelphia, PA 19104-6148. Tel.: (215) 898-0398. Fax: (215) 573-2000. E-mail: gdreyfuss@hhmi.upenn.edu

[1]*Abbreviations used in this paper:* BRCA1, breast cancer gene 1; Bru, bromouridine; CBP, cAMP response element binding protein; CTD, COOH-terminal domain; G/CB, nuclear accumulation containing gem- and coiled (cajal) body-specific marker; GST, glutathione *S*-transferase; hnRNP, heterogeneous nuclear RNP; IGC, interchromatin granule cluster; pol, polymerase; RHA, RNA helicase A; RNP, ribonucleoprotein; Sm, Smith antigen; snRNP, small nuclear RNP; SMA, spinal muscular atrophy; SMN, survival motor neuron; SR, serine- and arginine-rich; TBP, TATA box binding protein.

The pathway of spliceosomal snRNP assembly and function in higher eukaryotes requires a cytoplasmic phase where the snRNAs are exported from the nucleus, bind a common set of proteins to form the Sm core and, after 5′ cap hypermethylation and 3′ end maturation, are imported into the nucleus where snRNPs function in pre-mRNA splicing (Mattaj and De Robertis, 1985; Mattaj, 1986; Luhrmann et al., 1990). Experiments in *Xenopus laevis* oocytes and in mammalian cells have demonstrated a key function for the SMN complex in the cytoplasmic assembly of snRNPs (Fischer et al., 1997; Pellizzoni et al., 1998; Buhler et al., 1999). In addition, SMN functions in the nucleus in pre-mRNA splicing, a function which likely involves the regeneration of splicing components after rounds of splicing (Pellizzoni et al., 1998; Meister et al., 2000). SMN forms large oligomers, and this is essential for its activity, since a defect in SMN oligomerization correlates with SMA (Lorson et al., 1998; Pellizzoni et al., 1999). SMN oligomerization is necessary for efficient interaction with Sm proteins whereas SMN mutants found in SMA patients are deficient in both splicing regeneration activity and interaction with Sm proteins and with Gemin3 (Pellizzoni et al., 1998, 1999; Charroux et al., 1999; Friesen and Dreyfuss, 2000).

Here, we report the identification by nanoelectrospray mass spectrometry of RNA helicase A (RHA) as an SMN complex-interacting protein. In vitro, SMN interacts with RHA and this interaction is impaired for SMN mutants found in SMA patients. In vivo, the SMN complex is associated with RNA polymerase II (pol II), snRNPs, and RHA in a large complex. In the nucleus, the COOH-terminal domain (CTD) of pol II physically and functionally couples transcription, splicing, and polyadenylation (Steinmetz, 1997). We show that the SMN complex interacts with pol II CTD and this association is mediated, at least in part, by RHA. Expression of a dominant negative mutant of SMN (SMNΔN27) causes a dramatic reorganization of spliceosomal snRNPs into large nuclear accumulations which contain gem- and coiled body–specific markers (Pellizzoni et al., 1998). We show that expression of SMNΔN27 also causes the specific accumulation of the hypophosphorylated form of pol II (pol IIa) and RHA in these structures and inhibits transcription in vivo. Our findings suggest a role for the SMN complex in transcription, possibly for assembly of the key components of the mRNA transcription/processing machinery before their association with genes.

Materials and Methods

DNA Constructs

The cDNA encoding SMN was described previously (Liu and Dreyfuss, 1996). Plasmids expressing myc-tagged SMN wild-type and SMNΔN27 proteins under the control of the cytomegalovirus promoter were described previously (Pellizzoni et al., 1998). The RHA cDNA was a kind gift of Dr. Chee-Gun Lee (UMDNJ, New Jersey Medical School, Newark, NJ; Lee and Hurwitz, 1993).

Antibodies

Antibodies used in these experiments were as follows: mouse IgG1 monoclonal anti-SMN (2B1; Liu and Dreyfuss, 1996); mouse IgG1 monoclonal anti-Gemin2 (2E17; Liu et al., 1997); mouse IgG1 monoclonal anti-Gemin3 (11G9; Charroux et al., 1999); mouse IgG1 monoclonal anti-Gemin4 (22C10; Charroux et al., 2000); mouse IgG1 monoclonal antibromouridine (BrU) (Sigma-Aldrich); mouse IgG1 monoclonal anti-SC35 (Fu and Maniatis, 1990); mouse IgG3 monoclonal anti-Sm (Y12, Lerner et al., 1981); mouse IgG1 monoclonal anti-myc (9E10); affinity-purified rabbit polyserum anti-myc 9E10 epitope (A-14; Santa Cruz Biotechnology, Inc.); mouse IgM monoclonal anti-pol IIo (H5; Bregman et al., 1995; BabCo); mouse IgG2a monoclonal anti-pol IIa (8WG16; Thompson et al., 1989; BabCo); mouse IgG2a monoclonal anti-heterogeneous (hn)RNPA1 (4B10; Pinol-Roma et al., 1988); mouse IgG2b monoclonal anti-hnRNPC1/C2 (2B12; Dreyfuss et al., 1984); mouse IgG1 monoclonal anti-U2B′′ (4G3; Habets et al., 1989; Cappel); mouse IgG2a monoclonal anti-TFIIB (Transduction laboratories); rabbit polyserum anti–TATA box binding protein (TBP; Kato et al., 1994); and rabbit polyserum anti-RHA (Nakajima et al., 1997).

Cell Culture and Treatments

HeLa cells were cultured in DME (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL). HeLa cells, plated on glass coverslips, were transfected by the standard calcium phosphate method. After overnight incubation with DNA, cells were washed and fresh medium was added. Transfected cells were fixed and processed by immunofluorescence staining after an additional 24–36 h of incubation. Transfection efficiencies of myc-SMN wild-type and myc-SMNΔN27 plasmids were similar. Western blot analysis of lysates from transfected cells showed single bands of the expected size corresponding to myc-SMN wild-type and myc-SMNΔN27 proteins, the expression level of both proteins was approximately 10-fold over the endogenous SMN level (data not shown).

For in situ detection of sites of transcription, HeLa cells grown on glass coverslips were incubated for 1 h in culture medium containing 2 mM BrU (Sigma-Aldrich), washed briefly three times with PBS, fixed, and processed by immunofluorescence staining with anti-BrU monoclonal antibodies. RNase and DNase treatments were performed before immunofluorescence staining at room temperature in PBS containing 200 μg/ml RNase A and 100 μg/ml DNase I, respectively. To inhibit pol I or both pol I and pol II, HeLa cells were incubated for 3 h in the presence of either 0.04 or 5 μg/ml actinomycin D, respectively, before addition of BrU. To inhibit pol II activity, α-amanitin (50 μg/ml) was added to the culture medium 4 h before the addition of BrU.

Immunofluorescence Microscopy

Cells grown on glass coverslips were briefly washed with PBS, fixed in 2% formaldehyde PBS for 20 min at room temperature, permeabilized either in acetone for 3 min at −20°C or in 0.5% Triton X-100 PBS for 5 min at room temperature. Cells were blocked in PBS containing 3% BSA for 1 h at room temperature. Double-label immunofluorescence experiments were performed by separate sequential incubations of each primary antibody followed by incubation with its specific secondary antibody coupled to FITC or Texas red diluted in PBS–3% BSA. All incubations were at room temperature for 1 h. Laser confocal fluorescence microscopy was performed with a Leica TCS four-dimensional (Germany) confocal microscope. Images from each channel were recorded separately and then merged.

Immunoprecipitation Experiments

Immunoprecipitations were carried out using HeLa cell nucleoplasmic extract prepared in the presence of 0.5% Triton X-100 as described previously (Pinol-Roma et al., 1988). Immunoblotting was performed as described previously (Liu et al., 1997).

Protein Microsequencing by Mass Spectrometry

The p130 protein was coimmunoprecipitated from total HeLa cell extract with anti-Gemin3 monoclonal antibody (11G9), and the band was excised from a single one-dimensional Coomassie-stained polyacrylamide gel and in gel digested with trypsin as described previously (Shevchenko et al., 1996). Tryptic peptides were recovered from gel pieces by extraction with 5% formic acid and acetonitrile. The combined extracts were pooled, dried in a speed vac, and peptides were sequenced by nanoelectrospray tandem mass spectrometry (nano-ES MS/MS) as described in Wilm et al. (1996). The sequencing was performed on a PE-Sciex QSTAR instrument equipped with an ion source from Protana. Comprehensive protein and EST databases were searched using the Protana Software suite. No limitations on protein molecular weight and species of origin were imposed.

Recombinant Proteins Production

His-tagged SMN wild-type and mutant proteins cloned into pET28 and the glutathione *S*-transferase (GST) fusion of SMN and Gemin2 cloned into pGEX-5X were described previously (Pellizzoni et al., 1998, 1999). A GST–Gemin2 fusion construct, containing a tobacco etch virus protease cleavage site NH_2 terminal of Gemin2, in a pET21 vector carrying the

p15A origin of replication was kindly provided by Dr. Greg Van Duyne (University of Pennsylvania, Philadelphia, PA). GST–Gemin2 and his-tagged SMN wild-type or mutant proteins were coexpressed by cotransformation of the respective plasmids into *Escherichia coli* BL21(DE3) followed by induction with 1 mM IPTG at 17°C for 15 h. The recombinant proteins were purified by affinity chromatography on glutathione-Sepharose beads (Pharmacia) according to the manufacturer's instructions. The plasmid encoding the GST–CTD fusion protein was a generous gift of Dr. James Manley (Department of Biological Sciences, Columbia University, New York, NY). GST–CTD was produced in *E. coli* BL21(DE3) and purified as described above.

In Vitro Protein-binding Assay

The RHA full length (1–1279) and the RHAΔRGG COOH-terminal deletion (1–1106) proteins were produced by in vitro–coupled transcription/translation (Promega) in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech) using the RHA cDNA, either undigested or digested with Bgl II, respectively. Purified GST, GST–Gemin2, or GST–Gemin2/SMN proteins (2 μg) bound to 25 μl of glutathione-Sepharose beads were incubated with in vitro–translated protein products in 1 ml of binding buffer (50 mM, Tris-HCl, pH 7.5), 200 mM NaCl, 2 mM EDTA, 0.1% NP-40, 2 μg/ml leupeptin, pepstatin A, and aprotinin). After incubation for 1 h at 4°C, the beads were pelleted, washed five times with 1 ml of binding buffer, and boiled in SDS-PAGE sample buffer. The eluted proteins were analyzed by SDS-PAGE, and the signal was enhanced by treatment with Amplify solution (Amersham Pharmacia Biotech). Purified GST or GST–CTD bound to glutathione-Sepharose beads was incubated with either HeLa nuclear extract prepared according to Dignam et al. (1983), or [^{35}S]methionine-labeled in vitro–translated proteins in buffer D containing 0.025% NP-40 at 4°C. After five washes with the same buffer, bound proteins were eluted by boiling in SDS-PAGE, sample buffer, separated by SDS-PAGE and analyzed by Western blotting or autoradiography.

Results

Identification of RHA as an SMN-interacting Protein

We have recently identified two novel components of the SMN complex by a combination of coimmunoprecipitation and mass spectrometry (Charroux et al., 1999, 2000). Immunoprecipitations from [^{35}S]methionine-labeled HeLa cell lysates with antibodies against components of the SMN complex revealed the presence of additional proteins whose identity is not yet known (Liu et al., 1997; Charroux et al., 1999, 2000). Here, we employed the same strategy to identify a novel SMN complex-interacting protein. Fig. 1 A shows the proteins that specifically coimmunopurify with anti-Gemin3 antibodies compared with antitransportin antibodies as a control. In addition to the known components of the SMN complex (SMN, Gemin2, Gemin3, and Gemin4), there are protein bands of ~175, 130, and 95 kD. These bands were excised from the gel and digested with trypsin. The resulting peptides were sequenced by nano-electrospray mass spectrometry as described in Materials and Methods. In this paper, we report the identification of the 130-kD protein and experiments that led us to propose a role for the SMN complex in transcription. Databases search with several peptides revealed that p130 is identical to RHA, an essential ATP-dependent RNA helicase of the DEAH family (Lee and Hurwitz, 1993; Lee et al., 1998).

Because RHA contains an RGG box at the COOH terminus and SMN has been found to interact with some arginine- and glycine-rich domains (Liu and Dreyfuss, 1996; Friesen and Dreyfuss, 2000), we tested whether RHA can bind SMN in vitro. Fig. 1 B shows that in vitro–translated RHA binds efficiently to purified recombinant GST–SMN but not to GST–Gemin2 or GST alone. We also tested the ability of SMN mutants, expressed and purified as GST–Gemin2/SMN complexes, to bind RHA in vitro (Fig. 1 C). SMN interaction with RHA is impaired by either a single point mutation (SMNY272C) or a deletion of the amino acids encoded by exon 7 (SMNΔEx7) as found in some SMA patients. In contrast, the binding of SMNΔN27 to RHA is indistinguishable from that of SMN wild-type. Furthermore, the RGG-rich COOH-terminal domain of RHA, which is not required for its interactions with pol II and with transcriptional coactivators (Nakajima et al., 1997; Anderson et al., 1998), is necessary for interaction with SMN (Fig. 1, C and D).

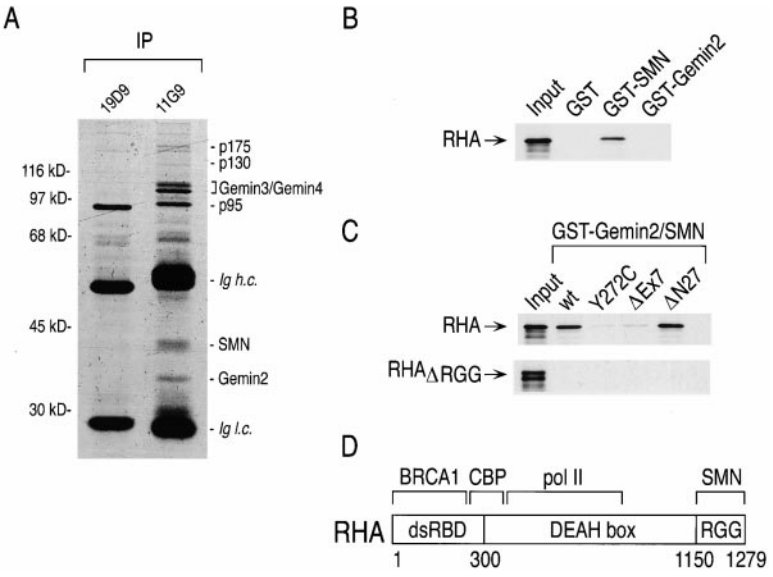

Figure 1. Identification of RHA as a novel SMN-interacting protein. (A) Coimmunoprecipitation experiments with anti-Gemin3 (11G9) or antitransportin (19D9) monoclonal antibodies were carried out from total HeLa cell extract. The immunoprecipitates (IP) were analyzed by SDS-PAGE and Coomassie blue staining. The band corresponding to the p130 protein coimmunoprecipitated with anti-Gemin3 antibodies was excised and identified by nanoelectrospray tandem mass spectrometry as described in Materials and Methods. Molecular weight markers, heavy (*Ig h.c.*) and light (*Ig l.c.*) chains of immunoglobulins, and the bands corresponding to the components of the SMN complex are indicated. (B) SMN interacts with RHA in vitro. In vitro–translated [^{35}S]methionine-labeled RHA full length (RHA) was incubated with either GST, GST–Gemin2, or GST–SMN as indicated. 10% of the input is shown. (C) The SMN interaction with RHA is impaired by SMN mutations found in SMA patients and requires the RGG-rich domain of RHA. In vitro–translated [^{35}S]methionine-labeled RHA full-length (RHA) or a COOH terminus deletion lacking the RGG domain (RHAΔRGG) were incubated with the indicated GST–Gemin2/SMN complexes containing either wild-type (wt) or mutant his-SMN proteins coexpressed and purified as described in Materials and Methods. 10% of the input is shown. (D) Schematic representation of the structure of RHA and the domains involved in the interaction with pol II, BRCA1, CBP, and SMN.

Association of the SMN Complex with Pol II, snRNPs, and RHA

We further examined the association between RHA and the SMN complex in vivo by coimmunoprecipitation experiments. Anti-RHA antibodies specifically coimmunoprecipitate SMN, Gemin2, Gemin3, and Gemin4 in addition to snRNPs and both hyperphosphorylated (pol IIo) and hypophosphorylated (pol IIa) pol II (Fig. 2). The general transcription factors TBP and transcription factor IIB are also detected in the RHA immunoprecipitate. None of these proteins are detectable in immunoprecipitates using a rabbit preimmune serum, and the abundant hnRNP A1 is not detected in the RHA immunoprecipitate. Fractionation of an HeLa nuclear extract by sucrose gradient centrifugation indicates that >30% of nuclear SMN, Gemin2, Gemin3, and Gemin4 cofractionate with RHA, snRNPs, and pol II as a large complex (Paushkin, S., and G. Dreyfuss, unpublished results). These results demonstrate that a large nuclear complex exists which contains pol II, snRNPs, RHA, and components of the SMN complex.

The CTD of pol II plays a key role in coupling transcription and pre-mRNA processing by interacting directly with components of the RNA processing machinery (for review see Steinmetz, 1997). To ask if the SMN complex can bind pol II CTD, we employed an approach similar to the one used to demonstrate the physical interaction of polyadenylation factors with pol II CTD (McCracken et al., 1997). We investigated whether pol II CTD can associate with the SMN complex by pull-down experiments from HeLa nuclear extract using GST–CTD. Fig. 3 A shows that SMN, Gemin2, Gemin3, and Gemin4 specifically associate with pol II CTD. The interaction of the SMN complex is not influenced by the phosphorylation state of the CTD (data not shown).

RHA has been suggested to function in transcription as a direct molecular link between pol II and transcriptional coactivators including cAMP response element binding protein (CBP)/p300 and breast cancer gene 1 (BRCA1; Nakajima et al., 1997; Anderson et al., 1998). We addressed the possibility that RHA may play a similar role in the association of SMN with pol II CTD. To do so, we incubated GST–CTD with in vitro–translated [^{35}S]methionine-labeled SMN in the presence or absence of similarly produced RHA and found that RHA stimulates the binding of SMN to pol II CTD (Fig. 3 B). Although these interactions may be mediated by additional components in the reticulocyte lysate or in the nuclear extract, the data strongly suggest that the SMN complex associates with pol II and

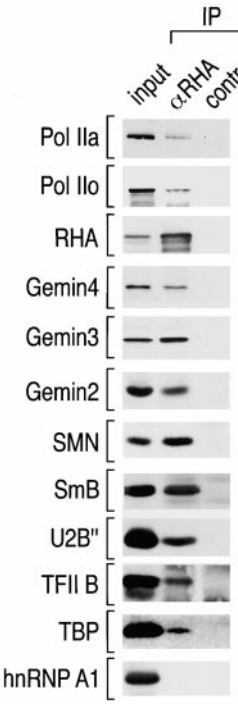

Figure 2. Association of the SMN complex with RHA in vivo. Immunoprecipitation (IP) experiments were carried out on HeLa cell nucleoplasmic extract using anti-RHA or control rabbit preimmune antibodies. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with the antibodies to the indicated proteins. 10% of the input is shown.

that RHA promotes this association possibly by stabilizing or acting as a bridging factor between pol II CTD and SMN.

Pol IIa Accumulates in the G/CBs

Previous transfection experiments in HeLa cells with SMNΔN27 showed that this dominant negative SMN mutant blocks snRNP assembly in the cytoplasm, causes a profound rearrangement of snRNPs in the nucleus, and inhibits pre-mRNA splicing in vitro (Pellizzoni et al., 1998). To address further the possible involvement of the SMN complex in transcription, we examined the subcellular localization of pol II in cells expressing SMNΔN27. Consistent with previous reports (Bregman et al., 1995), pol IIa shows a diffuse nucleoplasmic punctate distribution excluding nucleoli, and this is also the case in cells transfected with SMN wild-type (Fig. 4, A–C). However, in cells transfected with SMNΔN27, pol IIa is reorganized and accumulates in large nuclear aggregates which contain gem- and CB-specific markers (G/CBs [Fig. 4, D–F]). For brevity, we shall refer to these merged accumulations as G/CBs. The CTD of pol II undergoes extensive phosphorylation in vivo and pol IIo

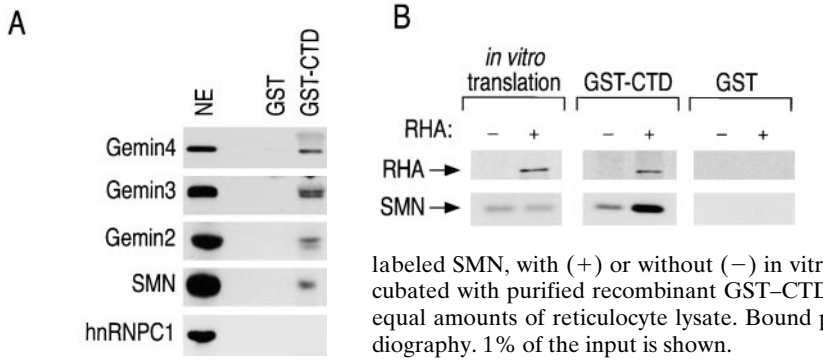

Figure 3. The SMN complex interacts with pol II CTD in vitro. (A) GST–CTD affinity chromatography. HeLa nuclear extract was incubated with either purified recombinant GST or GST–CTD, and bound proteins were analyzed by SDS-PAGE and Western blotting with antibodies to the indicated proteins. 2.5% of the input is shown. (B) RHA stimulates the interaction of SMN with pol II CTD. In vitro–translated [^{35}S]methionine-labeled SMN, with (+) or without (−) in vitro–translated [^{35}S]methionine-labeled RHA, was incubated with purified recombinant GST–CTD or GST as indicated. Binding reactions contained equal amounts of reticulocyte lysate. Bound proteins were analyzed by SDS-PAGE and autoradiography. 1% of the input is shown.

shows a speckled nucleoplasmic distribution more evident by confocal than by standard epifluorescence microscopy (Bregman et al., 1995; Zeng et al., 1997). The pattern of pol IIo distribution in cells transfected with SMN wild type, similar to untransfected cells, shows little or no localization in gems (Fig. 4, G–I). Surprisingly, on transfection with SMNΔN27, pol IIo is excluded from the large G/CBs, the diffuse nucleoplasmic pool appears reduced, and most of pol IIo is localized in interchromatin granule clusters (IGCs) around the G/CBs (Fig. 4, J–L; see Fig. 6, G–I).

RHA, TBP, and snRNPs Accumulate in the G/CBs

Next, we examined the effect of SMNΔN27 expression on the localization of RHA. RHA shows a nucleoplasmic distribution in untransfected HeLa cells and in cells transfected with SMN wild-type and with no evident colocalization with the G/CBs (Fig. 5, A–C). However, in cells transfected with SMNΔN27, RHA becomes relocalized in the nucleus and accumulates in the large G/CBs (Fig. 5, D–F). We also examined additional components of the pol II transcription/processing machinery such as TBP, polypyridimidine tract binding protein-associated splicing factor, and polyadenylation factors CstF64 and CPSF160, and found them to be similarly reorganized (Fig. 5, G–I; and data not shown). This reorganization of pol II, RHA, and TBP is restricted to the nuclear SMNΔN27-containing aggregates, whereas Sm proteins of spliceosomal snRNPs also accumulate in the cytoplasmic aggregates (Fig. 5, J–L), consistent with a role of the SMN complex in cytoplasmic snRNP assembly (Pellizzoni et al., 1998). In summary,

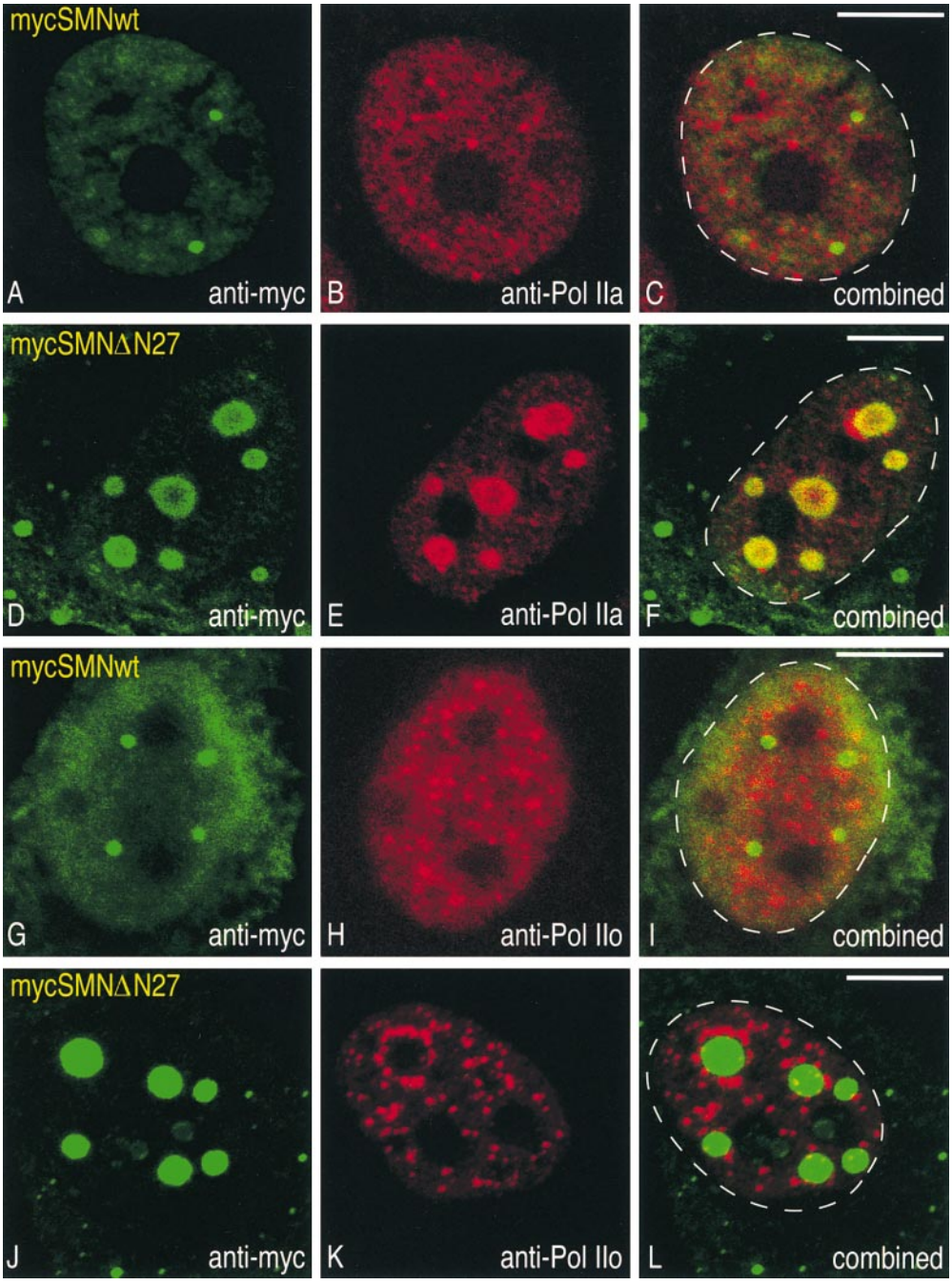

Figure 4. Expression of SMNΔN27 causes accumulation of pol IIa in G/CBs. (A–F) Double-label confocal immunofluorescence experiments using anti-myc (A and D), and anti-pol IIa (B and E) antibodies on HeLa cells transiently transfected with mycSMNwt (A–C) and mycSMNΔN27 (D–F). (G–L) Double-label confocal immunofluorescence experiments using anti-myc (G and J) and anti-pol IIo (H and K) antibodies on HeLa cells transiently transfected with mycSMNwt (G–I) and mycSMNΔN27 (J–L). The respective combined images are shown in C, F, I, and L. Colocalization results in a yellow signal. Dashed lines demarcate the nucleus. Bars, 5 μm.

several key components of the transcription and pre-mRNA processing machineries are strongly affected by SMNΔN27 expression and accumulate in the G/CBs.

Specificity of the Accumulation of Transcription and Pre-mRNA Processing Components in G/CBs

To assess the specificity of the accumulation in G/CBs of the components described above, we examined the effect of SMNΔN27 expression on hnRNP and serine- and arginine-rich (SR) proteins, two classes of protein that are both well characterized and intimately involved in the process of mRNA biogenesis. HnRNP proteins are very abundant nuclear RNA-binding proteins which interact with pre-mRNAs as they emerge from the transcription complex, modulate most of the posttranscriptional processes, and accompany the spliced mRNA to the cytoplasm (Dreyfuss et al., 1993). Fig. 6, A–F, shows that neither hnRNP A1 nor hnRNP C1/C2 proteins are reorganized by expression of SMNΔN27, and they are excluded from the G/CBs. Similarly, the essential splicing factor SC35, which is also a marker of IGCs (Spector, 1993), does not accumulate in the G/CBs (Fig. 6, G–I). Staining with another monoclonal antibody that reacts with the entire class of SR proteins shows a similar exclusion (Zahler et al., 1992; data not shown). Together these results demonstrate the specificity of the relocalization of components to G/CBs observed by expression of SMNΔN27. Nevertheless, SMNΔN27 expression alters the distribution of SC35. IGCs are found closely associated with the periphery of G/CBs, lose inter-

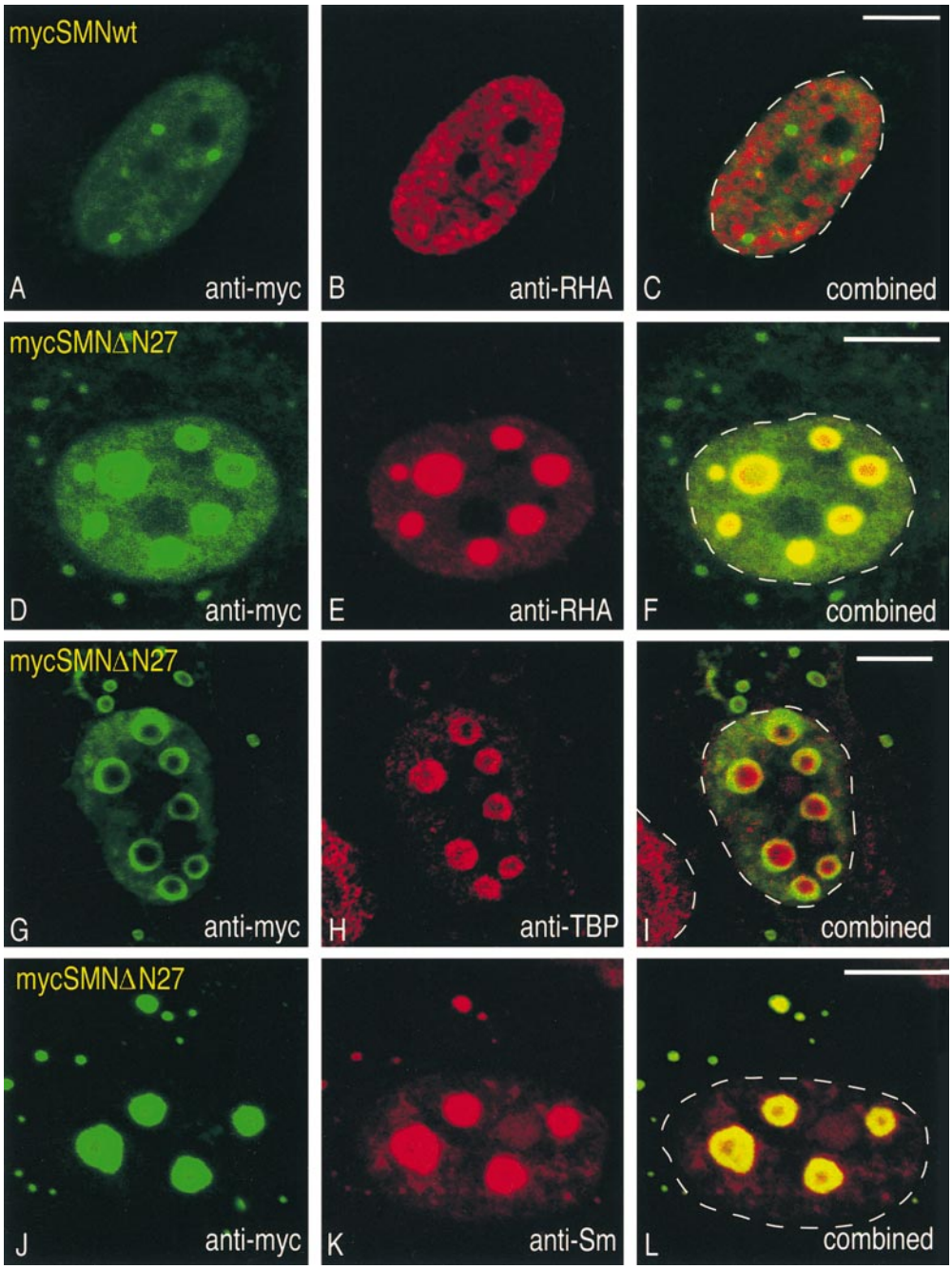

Figure 5. Expression of SMNΔN27 causes accumulation of RHA, TBP, and snRNPs in G/CBs. (A–F) Double-label confocal immunofluorescence experiments using anti-myc (A and D) and anti-RHA (B and E) antibodies on HeLa cells transiently transfected with mycSMNwt (A–C) and mycSMNΔN27 (D–F). (G–I) Double-label confocal immunofluorescence experiments using anti-myc (G) and anti-TBP (H) antibodies on HeLa cells transiently transfected with mycSMNΔN27. (J–L) Double-label confocal immunofluorescence experiments using anti-myc (J) and anti-Sm (K) antibodies on HeLa cells transiently transfected with mycSMNΔN27. The respective combined images are shown in C, F, I, and L. Colocalization results in a yellow signal. Dashed lines demarcate the nucleus. Bars, 5 μm.

connections with the surrounding nucleoplasm, and round up their contour in a way that resembles what is observed when transcription is inhibited by drugs or heat shock (Figs. 4 and 6; Zeng et al., 1997).

Expression of SMNΔN27 Inhibits Transcription In Vivo

Because of the strong effects of SMNΔN27, we were interested to determine whether transcription of pre-mRNAs in these cells is affected. The current methods for in situ detection of nascent RNA transcripts rely on microinjection of BrU triphosphate into living cells or run-on transcription in permeabilized cells followed by indirect immunofluorescence microscopy (Jackson et al., 1993; Wansink et al., 1993). By analogy with early experiments using [^{3}H]uridine incorporation for in vivo labeling of RNA (Fakan and Bernhard, 1971), we developed a simple method that avoids microinjection to detect transcriptional activity in living cells in situ (Fig. 7). An in situ method was necessary because only a fraction of the cells are transfected with SMNΔN27. We found that the addition of BrU to the culture medium results in its efficient uptake and incorporation into nascent RNA by the cells. Fig. 7 A shows the results of labeling HeLa cells for 1 h with 2 mM BrU followed by cell fixation and staining with anti-BrU antibodies. Pol II transcription sites are visible as anti-BrU reactive dots scattered throughout the nucleoplasm. Noticeably, nucleolar labeling, which is not readily detected by the methods of BrU triphosphate microinjection and run-on transcription in permeabilized cells (Jackson et al., 1993; Wansink et al., 1993), is also very intense. Higher concentrations of BrU do not increase the intensity of the signal but the intensity does increase proportional to the time of incubation (data not shown). The sensitivity of the staining to RNase treatment demonstrates that BrU was incorporated into RNA (Fig. 7 B). Although BrU may also be used as a thymidine analogue and become incorporated into DNA (Eidinoff et al., 1959), the staining we observed is resistant to DNase digestion, demonstrating that DNA incorporation does not significantly contribute to the signal detected (Fig. 7 C). Treatment with low concentrations of actinomycin D selectively inhibits transcription by pol I in the nucleolus (Fig. 7 D), whereas higher concentrations inhibit both pol I and pol II activities (Fig. 7 E). Extranucleolar incorporation is also strongly reduced by treatment with α-amanitin concentrations that inhibit pol II (Fig. 7 F). This method is rapid, simple, sensitive, and less invasive than previous methods, and is therefore probably more reflective of physiological transcription.

Using this method, we examined the transcriptional activity of cells transfected with SMN wild type or SMNΔN27.

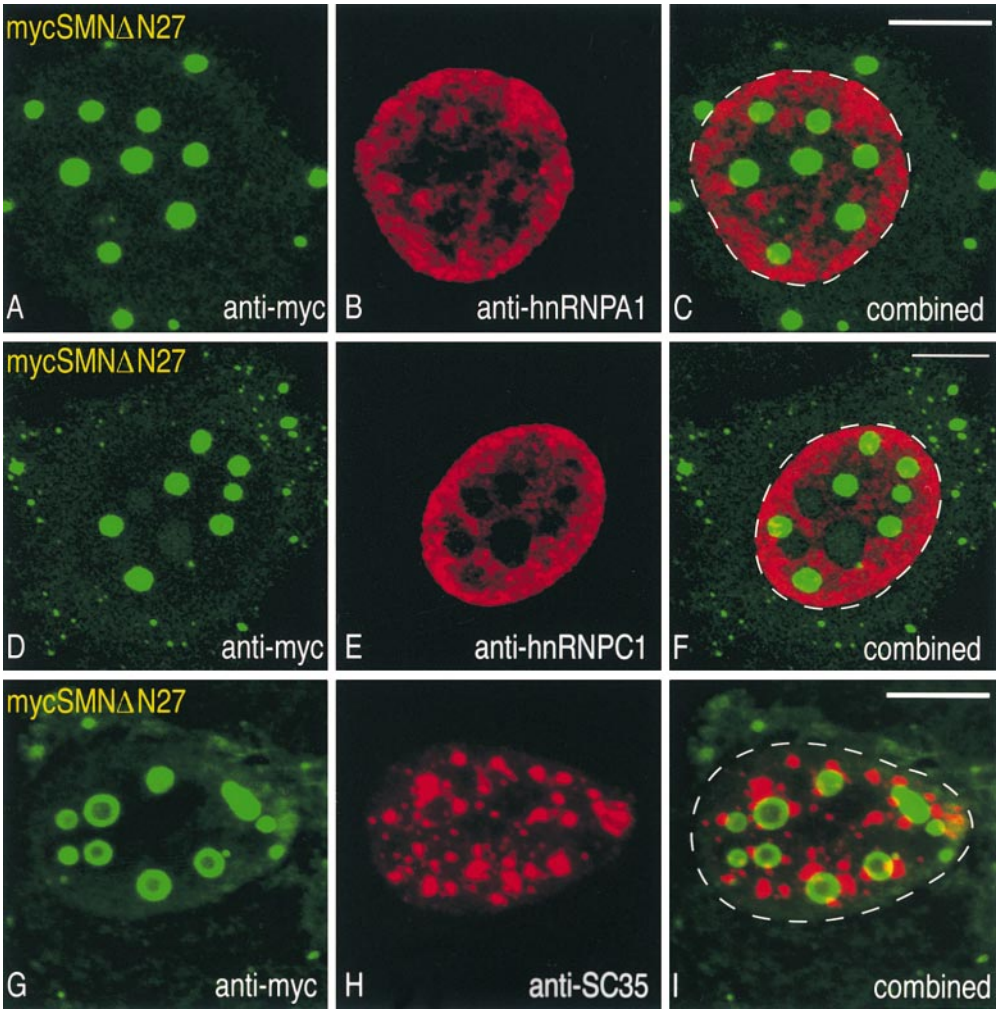

Figure 6. Specificity of the effects of SMNΔN27 expression. (A–I) Double-label confocal immunofluorescence experiments using anti-myc (A, D, and G) and either anti-hnRNP A1, anti-hnRNP C1/C2 or anti-SC35 antibodies (B, E, and H, respectively) on HeLa cells transiently transfected with mycSMNΔN27. The respective combined images are shown in C, F, and I. Dashed lines demarcate the nucleus. Bars, 5 μm.

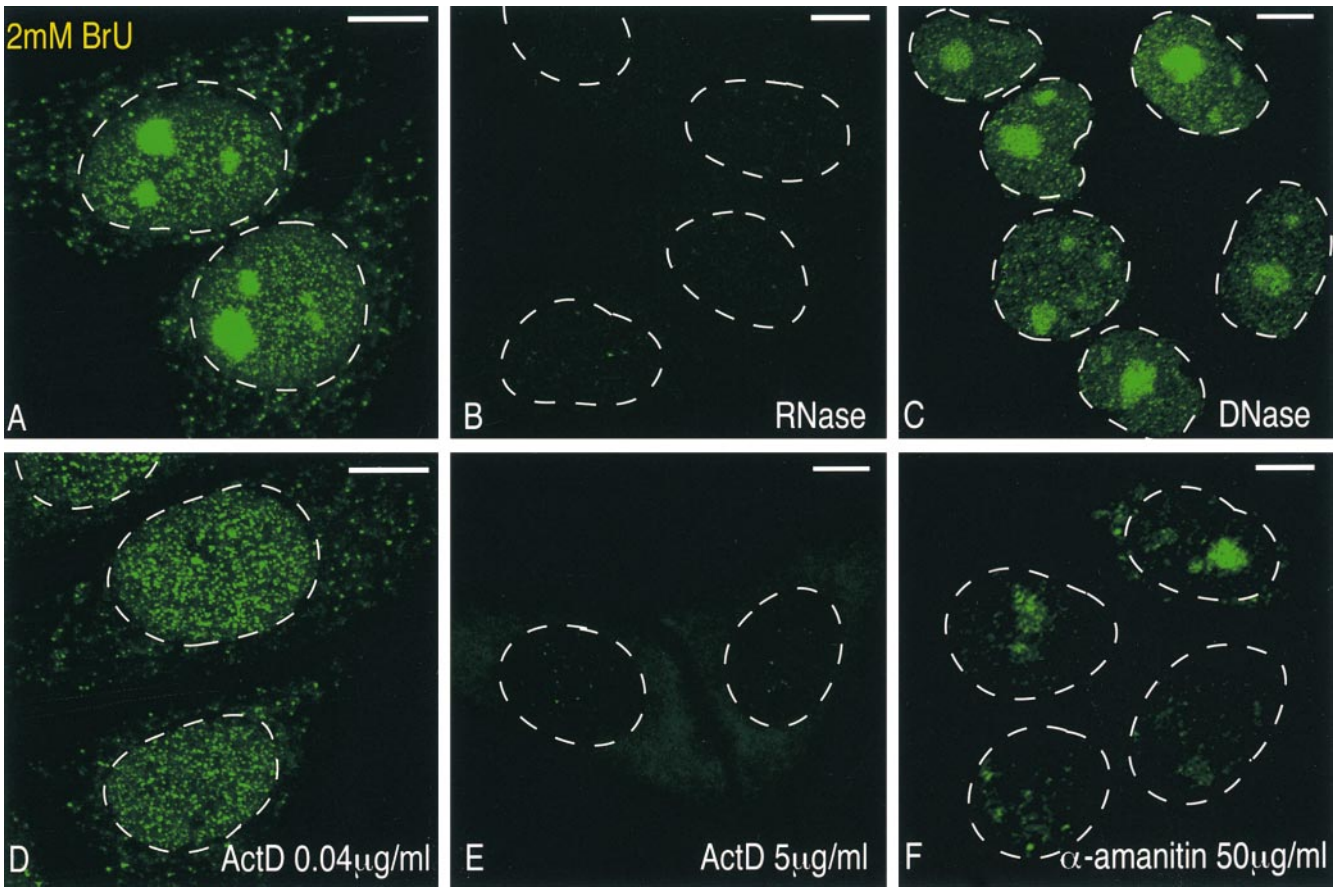

Figure 7. A rapid method for the in situ detection of transcription. (A) Indirect immunofluorescence detection of nascent RNA by laser confocal microscopy using anti-BrU antibodies on HeLa cells labeled in the presence 2 mM BrU as described in Materials and Methods. (B) Effect of RNase A treatment before immunostaining. (C) Effect of DNase I treatment before immunostaining. (D–E) Effect of low (0.04 μg/ml) or high (5 μg/ml) doses of actinomycin D (ActD) on BrU incorporation into RNA. (F) Effect of α-amanitin (50 μg/ml) on BrU incorporation into RNA. Dashed lines demarcate the nucleus. Bars, 5 μm.

Cells transfected with SMN wild-type, similar to untransfected cells, show high levels of transcription both in the nucleoplasm and in the nucleolus. No significant coincidence between SMN (or gem) staining with the sites of transcription was observed (Fig. 8, A–C). Strikingly, expression of SMNΔN27 strongly inhibits transcription both in the nucleoplasm and in the nucleolus, corresponding to pol II and pol I activities, respectively (Fig. 8, D–F). A reduction in the nucleoplasmic pool of pol II due to the accumulation of pol II in the G/CBs provides a likely explanation for the inhibition of transcription upon expression of SMNΔN27.

Discussion

We have identified RHA as a novel SMN-interacting protein and showed that this interaction is defective in SMN mutants found in some SMA patients. Coimmunoprecipitation and pull-down experiments demonstrated the physical association of the SMN complex with pol II, snRNPs, and RHA in vivo and in vitro. Importantly, the SMN complex binds pol II CTD, and RHA at least in part mediates this interaction. Thus, RHA likely plays a role in the association between the SMN complex and pol II possibly similar, but not mutually exclusive, to its role in bridging CBP and BRCA1 to pol II (Nakajima et al., 1997; Anderson et al., 1998). We further demonstrated that expression of SMNΔN27 brings about a profound rearrangement of pol II, RHA, snRNPs, TBP and polyadenylation factors, all of which specifically accumulate in enlarged G/CBs. This reorganization is accompanied by the inhibition of both pol I and pol II transcription, as detected in vivo by the BrU labeling method we have developed. These findings indicate a physical as well as a functional interaction between the SMN complex and components of the pol II transcription/processing machinery. Previous studies have indicated the presence of additional proteins associated with the SMN complex and the possibility that multiple SMN complexes may exist in vivo (Liu et al., 1997; Charroux et al., 1999, 2000; Meister et al., 2000). The observations that RHA does not localize to gems and is not found in all SMN complexes suggest that RHA is not a core component of the SMN complex such as Gemin2, Gemin3, and Gemin4. Similar to Sm proteins of spliceosomal snRNPs, we consider RHA as a possible substrate of SMN complex function.

The structure and organization of the nucleus are highly dynamic (Lamond and Earnshaw, 1998; Misteli and Spector, 1998; Lewis and Tollervey, 2000). Actively transcribing genes are found throughout the nucleus, and pol II transcriptional activity is not restricted to specific regions

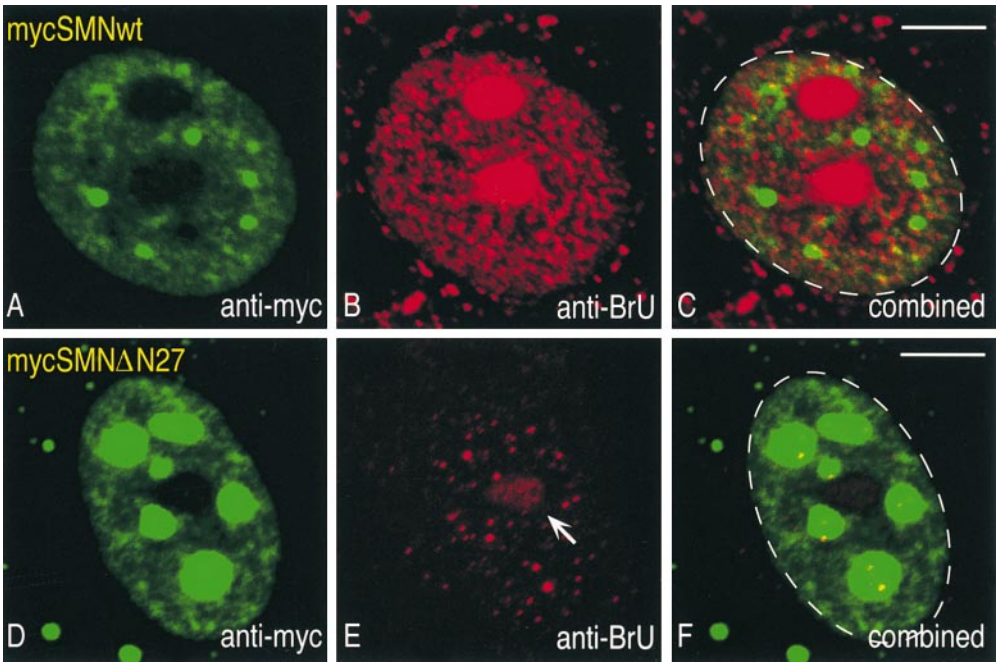

Figure 8. Expression of SMNΔN27 inhibits transcription by RNA pol I and II in vivo. (A–F) Double-label laser confocal microscopy of HeLa cells transiently transfected with mycSMNwt (A–C) and mycSMNΔN27 (D–F), showing the incorporation of BrU into RNA by indirect immunofluorescence using anti-myc (A and D) and anti-BrU (B and E) antibodies. BrU incorporation was performed as described in Materials and Methods on transfected cells before fixation and immunofluorescence staining. The respective combined images are shown in C and F. Colocalization results in a yellow signal. Dashed lines demarcate the nucleus. The nucleolus is indicated by an arrow in E. Bar, 5 μm.

(Jackson et al., 1993; Wansink et al., 1993). Since the initial observations of cotranscriptional splicing in vivo, considerable evidence has accumulated that most pre-mRNA processing steps take place coordinately at sites of transcription (Beyer and Osheim, 1988; Bauren and Wieslander, 1994). Physical and functional interactions between the transcriptional apparatus and the capping, splicing and polyadenylation machineries have recently been demonstrated, and pol II appears to be a key factor in coordinating all the major pre-mRNA processing events through its CTD (for review see Steinmetz, 1997; Neugebauer and Roth, 1997; Corden and Patturajan, 1998; Bentley, 1999; Hirose and Manley, 2000). Pol II CTD has been shown to associate with the capping enzyme, snRNPs, splicing and polyadenylation factors, possibly forming a large complex for which the terms "mRNA factory" or "transcriptosome" have been used (Halle and Meisterernst, 1996; McCracken et al., 1997; Corden and Patturajan, 1998). Recently, a role for CBs in the assembly of transcriptosomes has been suggested (Gall et al., 1999).

Our findings are consistent with the possibility that the assembly of transcriptosomes is a process that is extrinsic to, and precedes, binding of the polymerase complex to gene promoter elements. Most importantly, we propose that the SMN complex plays a central role in the pathway of transcriptosome assembly. The physical association of the SMN complex with components of the pol II transcription/processing machinery in untransfected cells indicates that they interact under normal conditions, and expression of SMNΔN27 likely blocks this pathway. Although no dominant–negative mutations have been reported in SMA patients, possibly because they would result in embryonic lethality, a point mutation in exon 1 has been identified in three unrelated SMA patients (Parsons et al., 1998). This suggests that the amino acid sequence encoded by exon 1, which is deleted in SMNΔN27, is important for SMN function and SMA. Moreover, we recently identified a dominant-negative mutant of Gemin2 that displays a phenotype similar to SMNΔN27 (Pellizzoni, L., and G. Dreyfuss, manuscript in preparation). The observation that expression of mutants of two different proteins of the SMN complex leads to the dramatic reorganization of components of the pol II transcription/processing machinery, and the inhibition of transcription in vivo argues that these effects result from disruption of a genuine pathway that requires SMN functions in normal cells.

Pol II CTD undergoes cycles of phosphorylation and dephosphorylation that modulate its association with processing factors and its activity in transcription (Dhamus, 1996). Pol IIa binds to the preinitiation complex on promoter elements, and CTD phosphorylation is believed to trigger release from the promoter and initiation of transcription (Lu et al., 1991; Akoulitchev et al., 1995). Pol IIo drives elongation and pre-mRNA processing events and is released from the gene upon termination of transcription (O'Brien et al., 1994). After their release from sites of transcription, pol II and several components of the transcription and pre-mRNA processing machineries seem to follow a common pathway in the nucleus (Zeng et al., 1997). A pool of both pol II isoforms exists in the nucleoplasm that is not engaged in active transcription and may represent the fraction of pol II undergoing recycling or regeneration between rounds of transcription (Kim et al., 1997; Zeng et al., 1997). Dephosphorylation of pol II CTD is one of the steps required for pol II recycling (Cho et al., 1999). The observation that the SMN complex associates with both pol II isoforms but only pol IIa accumulates in the G/CBs upon SMNΔN27 expression, suggests that pol II CTD dephosphorylation may take place in association with components of the SMN complex and that SMNΔN27 blocks the pathway downstream of this event.

HnRNP and SR proteins represent indirect markers of pre-mRNA distribution in the nucleus, and these abundant RNA-binding proteins do not accumulate in G/CBs (Fig. 6). Moreover, hnRNP proteins do not coimmunoprecipitate with the SMN complex (Fig. 2; data not shown).

These results suggest that the SMN complex associates with transcriptosome components that are not bound to pre-mRNAs. It is tempting to speculate that pol II and transcriptosome components associate with the SMN complex after their dissociation from transcribing genes and before being recruited for a new round of transcription.

TBP is a general transcription factor common to all three RNA polymerases (Hernandez, 1993; Rigby, 1993). TBP accumulates in the G/CBs upon expression of SMNΔN27, and likely as a consequence pol I transcription in the nucleolus is strongly inhibited. Similar to the case of pol II, RNA polymerase I holoenzyme as a transcriptionally competent large multisubunit complex has been identified, and the possibility that it may be preassembled in CBs has been suggested (Seither et al., 1998; Gall et al., 1999). By extension of what we have observed here for components of pol II transcriptosomes, the SMN complex may interact with components of the pol I transcription machinery and also play a role in the assembly of a class I transcriptosome. Experiments are underway to assess this possibility.

Our findings suggest a critical role for the SMN complex in several aspects of mRNA biogenesis. Neurons are likely to be particularly sensitive to defects in mRNA metabolism as neuronal differentiation and maintenance require highly active and finely regulated pre-mRNA transcription and processing activities (Tanabe and Jessell, 1996; Grabowski, 1998). It is conceivable that the drastic reduction in the amount of SMN in motor neurons of SMA patients leads to an impaired capacity to produce mRNAs, and thus leads to neuronal degeneration.

We thank Drs. Joan A. Steitz for anti-Sm, Yasutaka Makino and Takaaki Tamura for anti-TBP, Tom Maniatis for anti-SC35, and Jeffrey Parvin for anti-RHA antibodies. We also thank Drs. Chee-Gun Lee and James Manley for the RHA and GST–CTD constructs, respectively. We are grateful to members of our laboratory for stimulating discussions and, in particular, to Drs. Sara Nakielny, Westley Friesen, and Zissimos Mourelatos for helpful discussions and critical comments on this manuscript.

This work was supported by a grant from the National Institutes of Health. Work in M. Mann's laboratory was supported by a grant from the Danish National Research Foundation. G. Dreyfuss is an Investigator of the Howard Hughes Medical Institute.

Submitted: 13 September 2000
Revised: 26 October 2000
Accepted: 14 November 2000

References

Akoulitchev, S., T.P. Makela, R.A. Weinberg, and D. Reinberg. 1995. Requirement for TFIIH kinase activity in transcription by RNA polymerase II. *Nat*ure. 377:557–560.

Anderson, S.F., B.P. Schlegel, T. Nakajima, E.S. Wolpin, and J.D. Parvin. 1998. BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nat. Genet.* 19:254–256.

Bauren, G., and L. Wieslander. 1994. Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. *Cell.* 76:183–192.

Bentley, D. 1999. Coupling RNA polymerase II transcription with pre-mRNA processing. *Curr. Opin. Cell Biol.* 11:347–351.

Beyer, A.L., and Y.N. Osheim. 1988. Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.* 2:754–765.

Bregman, D.B., L. Du, S. van der Zee, and S.L. Warren. 1995. Transcription-dependent redistribution of the large subunit RNA polymerase II to discrete nuclear domains. *J. Cell Biol.* 129:287–298.

Buhler, D., V. Raker, R. Luhrmann, and U. Fischer. 1999. Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. *Hum. Mol. Genet.* 8:2351–2357.

Burghes, A.H.M. 1997. When is a deletion not a deletion? When it is converted. *Am. J. Hum. Genet.* 61:9–15.

Charroux, B., L. Pellizzoni, R.A. Perkinson, A. Shevchenko, M. Mann, and G. Dreyfuss. 1999. Gemin3: a novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J. Cell Biol.* 147:1181–1193.

Charroux, B., L. Pellizzoni, R.A. Perkinson, J. Yong, A. Shevchenko, M. Mann, and G. Dreyfuss. 2000. Gemin4: a novel component of the SMN complex that is found in both gems and nucleoli. *J. Cell Biol.* 148:1177–1186.

Cho, H., T.K. Kim, H. Mancebo, W.S. Lane, O. Flores, and D. Reinberg. 1999. A protein phosphatase functions to recycle RNA polymerase II. *Genes Dev*. 13:1540–1552.

Coovert, D., T.T. Le, P.E. McAndrew, J. Strasswimmer, T.O. Crawford, J.R. Mendell, S.E. Coulson, E.J. Androphy, T.W. Prior, and A.H.M. Burghes. 1997. The survival motor neuron protein in spinal muscular atrophy. *Hum. Mol. Genet.* 6:1205–1214.

Corden, J.L., and M. Patturajan. 1998. A CTD function linking transcription to splicing. *Trends Biochem. Sci.* 22:413–416.

Dhamus, M.E. 1996. Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J. Biol. Chem*. 271:19009–19012.

Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475–1489.

Dreyfuss, G., Y.D. Choi, and S.A. Adam. 1984. Characterization of heterogeneous nuclear RNA-protein complexes in vivo with monoclonal antibodies. *Mol. Cell. Biol*. 4:1104–1114.

Dreyfuss, G., M.J. Matunis, S. Pinol-Roma, and C. Burd. 1993. HnRNP proteins and the biogenesis of the mRNA. *Annu. Rev. Biochem*. 62:289–321.

Eidinoff, M.L., J.E. Knoll, B.J. Marano, and D. Klein. 1959. Pyrimidine studies. Effect of 5-bromodeoxyuridine and related nucleosides on incorporation of precursors into nucleic acid pyrimidines. *Cancer Res*. 19:738–745.

Fakan, S., and W. Bernhard. 1971. Localisation of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. *Exp. Cell Res*. 67:129–141.

Fischer, U., Q. Liu, and G. Dreyfuss. 1997. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell.* 90:1023–1029.

Friesen, W.J., and G. Dreyfuss. 2000. Specific sequences of the Sm and Sm-like (Lsm) proteins mediate their interaction with the spinal muscular atrophy disease gene product (SMN). *J. Biol. Chem*. 275:26370–26375.

Frugier, T., F.D. Tiziano, C. Cifuentes-Diaz, P. Miniou, N. Roblot, A. Dierich, M. Le Meur, and J. Melki. 2000. Nuclear targeting defect of SMN lacking the C-terminus in a mouse model for spinal muscular atrophy. *Hum. Mol. Genet.* 9:849–858.

Fu, X.D., and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature.* 343:437–441.

Gall, J.G., M. Bellini, Z. Wu, and C. Murphy. 1999. Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. *Mol. Biol. Cell.* 10:4385–4402.

Grabowski, P.J. 1998. Splicing regulation in neurons: tinkering with cell-specific control. *Cell.* 92:709–712.

Habets, W.J., M.H. Hoet, B.A. De Jong, A. Van der Kemp, and W.J. Van Venrooij. 1989. Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. *J. Immunol*. 143:2560–2566.

Halle, J.P., and M. Meisterernst. 1996. Gene expression: increasing evidence for a transcriptosome. *Trends Genet*. 12:161–163.

Hannus, S., D. Buhler, M. Romano, B. Seraphin, and U. Fischer. 2000. The *Schizosaccharomyces pombe* protein Yab8p and a novel factor, Yip1p, share structural and functional similarity with the spinal muscular atrophy-associated proteins SMN and SIP1. *Hum. Mol. Genet*. 9:663–674.

Hernandez, N. 1993. TBP, a universal eukaryotic transcription factor? *Genes Dev*. 7:1291–1308.

Hirose, Y., and J.L. Manley. 2000. RNA polymerase II and the integration of nuclear events. *Genes Dev*. 14:1415–1429.

Hsieh-Li, H.M., J.G. Chang, Y.J. Jong, M.H. Wu, N.M. Wang, C.H. Tsai, and H. Li. 2000. A mouse model for spinal muscular atrophy. *Nat. Genet*. 24:66–70.

Jablonka, S., B. Schrank, M. Kralewski, W. Rossol, and M. Sendtner. 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.

Jackson, D.A., A.B. Hassan, R.J. Errington, and P.R. Cook. 1993. Visualization of focal sites of transcription within human nuclei. *EMBO (Eur. Mol. Biol. Organ.) J*. 12:1059–1065.

Kato, K., Y. Makino, T. Kishimoto, J. Yamauchi, S. Kato, M. Muramatsu, and T. Tamura. 1994. Multimerization of the mouse TATA-binding protein (TBP) driven by its C-terminal conserved domain. *Nucleic Acids Res.* 22: 1179–1185.

Kim, E., L. Du, D.B. Bregman, and S.L. Warren. 1997. Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA. *J. Cell Biol.* 136:19–28.

Lamond, A.I., and W.C. Earnshaw. 1998. Structure and function in the nucleus. *Science.* 280:547–553.

Lee, C.G., and J. Hurwitz. 1993. Human RNA helicase A is homologous to the maleless protein of *Drosophila*. *J. Biol. Chem*. 268:16822–16830.

Lee, C.G., V. Da Costa Soares, C. Newberger, K. Manova, E. Lacy, and J. Hurwitz. 1998. RNA helicase A is essential for normal gastrulation. *Proc. Natl. Acad. Sci. USA*. 95:13709–13713.

Lefebvre, S., L. Burglen, S. Reboullet, O. Clermont, P. Burlet, L. Viollet, B.

Benichou, C. Cruaud, P. Millasseau, M. Zeviani, et al. 1995. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* 89:155–165.

Lefebvre, S., P. Burlet, Q. Liu, S. Bertrandy, O. Clermont, A. Munnich, G. Dreyfuss, and J. Melki. 1997. Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.* 16:265–269.

Lerner, E.A., M.R. Lerner, J.A. Hardin, C.A. Janeway, Jr., and J.A. Steitz. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 78:2737–2741.

Lewis, J.D., and D. Tollervey. 2000. Like attracts like: getting RNA processing together in the nucleus. *Science.* 288:1385–1389.

Liu, Q., and G. Dreyfuss. 1996. A novel nuclear structure containing the survival of motor neuron proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:3555–3565.

Liu, Q., U. Fischer, F. Wang, and G. Dreyfuss. 1997. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell.* 90:1013–1021.

Lorson, C.L., J. Strasswimmer, J.M. Yao, J.D. Baleja, E. Hahnen, B. Wirth, T. Le, A.H.M. Burghes, and E. Androphy. 1998. SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nat. Genet.* 19:63–66.

Lorson, C.L., E. Hahnan, E.J. Androphy, and B. Wirth. 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.

Lu, H., O. Flores, R. Weinmann, and D. Reinberg. 1991. The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci USA* 88:10004–10008.

Luhrmann, R., B. Kastner, and M. Bach. 1990. Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochem. Biophys. Acta.* 1087: 265–292.

Mattaj, I.W. 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell.* 46:905–911.

Mattaj, I.W., and E.M. De Robertis. 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. *Cell.* 40:111–118.

McCracken, S., N. Fong, K. Yankulov, S. Ballantyne, G. Pan, J. Greenblatt, S.D. Patterson, M. Wickens, and D.L. Bentley. 1997. The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. *Nature.* 385:357–361.

Meister, G., D. Buhler, B. Laggerbauer, M. Zobawa, F. Lottspeich, and U. Fischer. 2000. Characterization of a nuclear 20S complex containing the survival of motor neurons (SMN) protein and a specific subset of spliceosomal Sm proteins. *Hum. Mol. Genet.* 9:1977–1986.

Melki, J. 1997. Spinal muscular atrophy. *Curr. Opin. Neurol.* 10:381–385.

Miguel-Aliaga, I., E. Culetto, D.S. Walker, H.A. Baylis, D.B. Sattelle, and K.E. Davies. 1999. The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum. Mol. Genet.* 8:2133–2143.

Misteli, T., and D.L. Spector. 1998. The cellular organization of gene expression. *Curr. Opin. Cell Biol.* 10:323–331.

Monani, U.R., M. Sendtner, D.D. Coovert, D.W. Parsons, C. Andreassi, T.T. Le, S. Jablonka, B. Schrank, W. Rossol, T.W. Prior, G.E. Morris, and A.H.M. Burghes. 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.

Nakajima, T., C. Uchida, S.F. Anderson, C.G. Lee, J. Hurwitz, J. Parvin, and M. Montminy. 1997. RNA helicase A mediates association of CBP with RNA polymerase II. *Cell.* 90:1107–1112.

Neugebauer, K.M., and M.B. Roth. 1997. Transcription units as RNA processing units. *Genes Dev.* 11:3279–3285.

O'Brien, T., S. Hardin, A. Greenleaf, and J.T. Lis. 1994. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. *Nature.* 370:75–77.

Owen, N., C.L. Doe, J. Mellor, and K.E. Davies. 2000. Characterization of the *Schizosaccharomyces pombe* orthologue of the human survival motor neuron (SMN) protein. *Hum. Mol. Genet.* 9:675–684.

Parsons, D.W., P.E. McAndrew, S.T. Iannaccone, J.R. Mendell, A.H.M. Burghes, and T.W. Prior. 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.

Paushkin, S., B. Charroux, L. Abel, R.A. Perkinson, L. Pellizzoni, and G. Dreyfuss. 2000. The survival motor neuron protein of *Schizosaccharomyces pombe*. Conservation of survival motor neuron interaction domains in divergent organisms. *J. Biol. Chem.* 275:23841–23846.

Pearn, J. 1980. Classification of spinal muscular atrophies. *Lancet.* 1:919–922.

Pellizzoni, L., N. Kataoka, B. Charroux, and G. Dreyfuss. 1998. A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell.* 95:615–624.

Pellizzoni, L., B. Charroux, and G. Dreyfuss. 1999. SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins. *Proc. Natl. Acad. Sci. USA.* 96:11167–11172.

Pinol-Roma, S., Y.D. Choi, M.J. Matunis, and G. Dreyfuss. 1988. Immunopurification of heterogeneous nuclear ribonucleoprotein particles reveals an assortment of RNA-binding proteins. *Genes Dev.* 2:215–227.

Rigby, P.W. 1993. Three in one and one in three: it all depends on TBP. *Cell.* 72:7–10.

Schrank, B., R. Gotz, J.M. Gunnersen, J.M. Ure, K.V. Toyka, A.G. Smith, and M. Sendtner. 1997. Inactivation of the survival of motor neuron gene, a candidate gene for human muscular atrophy, leads to massive cell death in early mouse embryos. *Proc. Natl. Acad. Sci. USA.* 94:9920–9925.

Seither, P., S. Iben, and I. Grummt. 1998. Mammalian RNA polymerase I exists as a holoenzyme with associated basal transcription factors. *J. Mol. Biol.* 275: 43–53.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* 68:850–858.

Spector, D.L. 1993. Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.* 9:265–315.

Steinmetz, E.J. 1997. Pre-mRNA processing and the CTD of RNA polymerase II: the tail that wags the dog? *Cell.* 89:491–494.

Tanabe, Y., and T.M. Jessell. 1996. Diversity and pattern in the developing spinal cord. *Science.* 274:1115–1123.

Thompson, N.E., T.H. Steinberg, D.B. Aronson, and R.R. Burgess. 1989. Inhibition of in vivo and in vitro transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptapeptide repeat of eukaryotic RNA polymerase II. *J. Biol. Chem.* 264:11511–11520.

Wansink, D.G., W. Schul, I. van der Kraan, B. van Steensel, R. van Driel, and L. de Jong. 1993. Fluorescent labelling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J. Cell Biol.* 122:282–293.

Wilm, M., A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, and M. Mann. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano electrospray mass spectrometry. *Nature.* 379:466–469.

Zahler, A.M., W.S. Lane, J.A. Stolk, and M.B. Roth. 1992. S/R proteins—a conserved family of pre-messenger-RNA splicing factors. *Genes Dev.* 6:837–847.

Zeng, C., E. Kim, S.L. Warren, and S.M. Berget. 1997. Dynamic relocation of transcription and splicing factors dependent upon transcriptional activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:1401–1412.