JCB Article

Symmetrical dimethylarginine methylation is required for the localization of SMN in Cajal bodies and pre-mRNA splicing

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The nuclear structures that contain symmetrical dimethylated arginine (sDMA)–modified proteins and the role of this posttranslational modification is unknown. Here we report that the Cajal body is a major epitope in HeLa cells for an sDMA-specific antibody and that coilin is an sDMA-containing protein as analyzed by using the sDMA-specific antibody and matrix-assisted laser desorption ionization time of flight mass spectrometry. The methylation inhibitor 5′-deoxy-5′-methylthioadenosine reduces the levels of coilin methylation and causes the appearance of SMN-positive gems. In cells devoid of Cajal bodies, such as primary fibroblasts, sDMA-containing proteins concentrated in speckles. Cells from a patient with spinal muscular atrophy, containing low levels of the methyl-binding protein SMN, localized sDMA-containing proteins in the nucleoplasm as a discrete granular pattern. Splicing reactions are efficiently inhibited by using the sDMA-specific antibody or by using hypomethylated nuclear extracts, showing that active spliceosomes contain sDMA polypeptides and suggesting that arginine methylation is important for efficient pre-mRNA splicing. Our findings support a model in which arginine methylation is important for the localization of coilin and SMN in Cajal bodies.

Introduction

Protein arginine methylation is a posttranslational modification that results in the mono- and dimethylation of the guanidino nitrogen atoms of arginine (Gary and Clarke, 1998). Arginines can be dimethylated either in a symmetrical or asymmetrical manner (symmetrical dimethylated arginine [sDMA]* and asymmetrical dimethylated arginine [aDMA]). Three of the six identified protein arginine methyltransferases (PRMT1, PRMT5, and PRMT6) catalyze the formation of dimethylated arginines in glycine- and arginine-rich regions (GARs) (Gary and Clarke, 1998; Frankel et al., 2001). The two major groups of proteins that contain aDMA include RNA-binding proteins and histones (McBride and Silver, 2001). Asymmetrical dimethylation of arginines has been shown to influence nuclear export (Lee et al., 1996), nuclear import (Xu et al., 2001), protein–protein interactions (Bedford et al., 2000), and transcription (Chen et al., 1999; Yun and Fu, 2000; Mowen et al., 2001; Wang et al., 2001). In contrast, much less is known about the role of sDMAs in cellular processes. PRMT5, the only known PRMT to catalyze sDMA (Branscombe et al., 2001), has been shown to methylate myelin basic protein (MBP) and histones on arginines in vitro (Pollack et al., 1999). PRMT5 resides in a cytoplasmic 20S complex with pICln and also has methyltransferase activity toward SmB, B′, D1, and D3 proteins (Brahms et al., 2001; Friesen et al., 2001b; Meister et al., 2001b). MBP, SmB, B′, D1, and D3, and the Sm-like protein LSm4 are the only known proteins that contain sDMAs in vivo (Baldwin and Carnegie, 1971; Brahms et al., 2000, 2001).

The cellular localization of sDMA-containing proteins is unknown, but these proteins have been proposed to be imported

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*Abbreviations used in this paper: aDMA, asymmetrical dimethylated arginine; AdML, adenovirus major late; GAR, glycine- and arginine-rich region; IGC, interchromatin granule cluster; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MBP, myelin basic protein; MTA, 5′-deoxy-5′-methylthioadenosine; NRP, ribonucleoprotein; sDMA, symmetrical dimethylated arginine; siRNA, small interfering RNA; SMA, spinal muscular atrophy.

Key words: PRMT5; Cajal; SMN; arginine methylation; splicing

within the nucleus by a complex that contains the sDMA-binding protein, SMN (Friesen et al., 2001a). SMN is the protein product of the survival of motor neurons gene (*SMN1*) responsible for spinal muscular atrophy (SMA) (Lefebvre et al., 1995). SMA is an autosomal recessive neurodegenerative disease, which is characterized at the clinical level by degeneration and loss of spinal cord motor neurons resulting in muscular weakness and atrophy (Melki, 1997). Patients with SMA have a marked decrease in the levels of SMN protein (Burghes, 1997; Coovert et al., 1997) and contain mutations that disrupt associations with its arginine glycine-rich–binding partners (Pellizzoni et al., 1999). SMN protein complexes function in the assembly of various ribonucleoprotein (RNP) complexes including U snRNPs (Fischer et al., 1997; Pellizzoni et al., 1998; Meister et al., 2001a). The role of arginine methylation in the process of RNP complex assembly is unknown, but it is thought to be one of the signals for targeting to the SMN complexes (Friesen et al., 2001b; Meister et al., 2001b).

The nucleus contains many dynamic nuclear structures, including Cajal (coiled) bodies, gems, interchromatin granule clusters (IGCs), the perinucleolar compartment, PML bodies, Sam68 nuclear bodies, and the nucleolus (Lamond and Earnshaw, 1998; Matera, 1999; Gall, 2000; Spector, 2001). Although the function of the Cajal body remains unknown, there is considerable evidence suggesting that it may be involved in snRNP maturation/biogenesis, histone pre-mRNA processing, and the assembly of the transcriptosomes (Matera, 1999; Gall, 2000). The Cajal body contains many components including coilin (the marker of Cajal bodies [Andrade et al., 1993]), snRNPs (Eliceiri and Ryerse, 1984; Fakan et al., 1984), and SMN (Liu and Dreyfuss, 1996; Pellizzoni et al., 1998). Cajal bodies have a twin structure called gems that contain SMN complexes but not snRNPs or coilin (Liu and Dreyfuss, 1996). It is not known why only a subset of cells contain gems. Coilin associates with SMN and requires its arginine glycine–rich regions (Hebert et al., 2001).

Herein we show that Cajal bodies are rich in sDMA-containing proteins in HeLa cells and that coilin is a methylated protein. In primary cells devoid of Cajal bodies, sDMA proteins stained nuclear speckles. Interestingly, in cells derived from a patient with SMA, which express very low levels of SMN, sDMA-containing proteins are located in a granular pattern within the nucleus. Our results suggest that the levels of the methyl-binding protein SMN may regulate the nuclear structures that are recognized by methyl-specific antibodies in situ. Moreover, we show data suggesting that a normal level of sDMA-containing proteins in the cells is important for pre-mRNA splicing.

Results

A novel anti-sDMA–specific antibody

Symmetrical dimethylation of arginines is a posttranslational modification that has been described for MBPs (Baldwin and Carnegie, 1971), the SmB, B′, D1, D3, and LSm4 proteins (Brahms et al., 2000, 2001). To study the role of symmetrical dimethylated arginines, an sDMA-specific antibody was generated by immunizing rabbits with a KLH-coupled nonapeptide containing four sDMA-glycine repeats. The affinity-purified antibody, named SYM10, recognized peptides corresponding to the COOH termini of Sm proteins D1 and D3 containing sDMA (SmD1 and SmD3 sDMA [Fig. 1 A]) and a generic RG-rich peptide containing sDMA (GAR sDMA [Fig. 1 A]). The SYM10 antibody did not recognize nonmethylated peptides corresponding to SmD3, GAR, and an unrelated RG-rich sequence (RG [Fig. 1 A]) or peptides containing aDMA (GAR aDMA [unpublished data; Fig. 1 A]). The SYM10 antibody had the strongest affinity for the immunizing peptide sym10^{sDMA}. SYM10 did not recognize a peptide from MBP that contained a unique sDMA-glycine repeat (MBP sDMA [Fig. 1 A]). The fact that SYM10 did not recognize the methylated MBP peptide suggests that SYM10 requires more than one sDMA-G dipeptide in a given polypeptide for binding. To better characterize the epitope recognized by SYM10, we performed ELISAs using a series of synthetic nonapeptides based on the immunizing peptide (sym10) backbone (Fig. 1 B). Even at a 1:500 dilution, the SYM10 antibody did not show any reactivity for the nonmethylated sym10 peptide or for a sym10 derivative containing only one sDMA residue (Fig. 1 B, asterisk and diamond, respectively). Interestingly, SYM10 reacted more strongly with a derivative harboring two spaced sDMA-Gs than two contiguous sDMA-G dipeptides (Fig. 1 B, triangle and circle, respectively). This defines the SYM10 epitope as requiring at least two, preferentially noncontiguous, sDMA-Gs in a given polypeptide sequence.

SYM10 recognizes many cellular proteins including coilin and the Sm proteins B, B′, and D

Immunoprecipitations were performed to identify the endogenous proteins that contain sDMA at steady state. HeLa cells were metabolically labeled with (^{3}H-*methyl*)-L-methionine in the presence of cycloheximide and chloramphenicol for 3 h, an established assay to measure in vivo methylation (Liu and Dreyfuss, 1995). Translational inhibitors were used to ensure that incorporation of the (^{3}H-*methyl*) group occurred only as a result of methylation. Cells were lysed, and immunoprecipitations were performed using the SYM10 antibody, the snRNP-specific antibody Y12 (Lerner et al., 1981), or normal rabbit serum (Fig. 1 C). The bound proteins were resolved by electrophoresis and visualized by fluorography. SYM10 immunoprecipitated a pattern of methylated proteins that was similar but distinguishable from the snRNP antibody Y12 (Fig. 1 C, lane 1 compared with 2). Since SYM10 recognized methylated SmB′, B, and D proteins, we examined whether SYM10 could immunoprecipitate other unmethylated Sm proteins as part of a complex. HeLa cells were metabolically labeled with ^{35}S-methionine and immunoprecipitated by using SYM10 or Y12. Both antibodies immunoprecipitated the complete repertoire of Sm proteins (Fig. 1 D). These findings imply that SYM10 recognizes methylated SmB′, B, and D in the context of mature snRNP complexes. SYM10 also immunoprecipitated a unique pattern of higher molecular weight polypeptides that was distinct from Y12 (Fig. 1 D). To determine which proteins are recognized directly by SYM10, immunoblotting was performed on HeLa total cell extracts (Fig. 1 E). As ex-

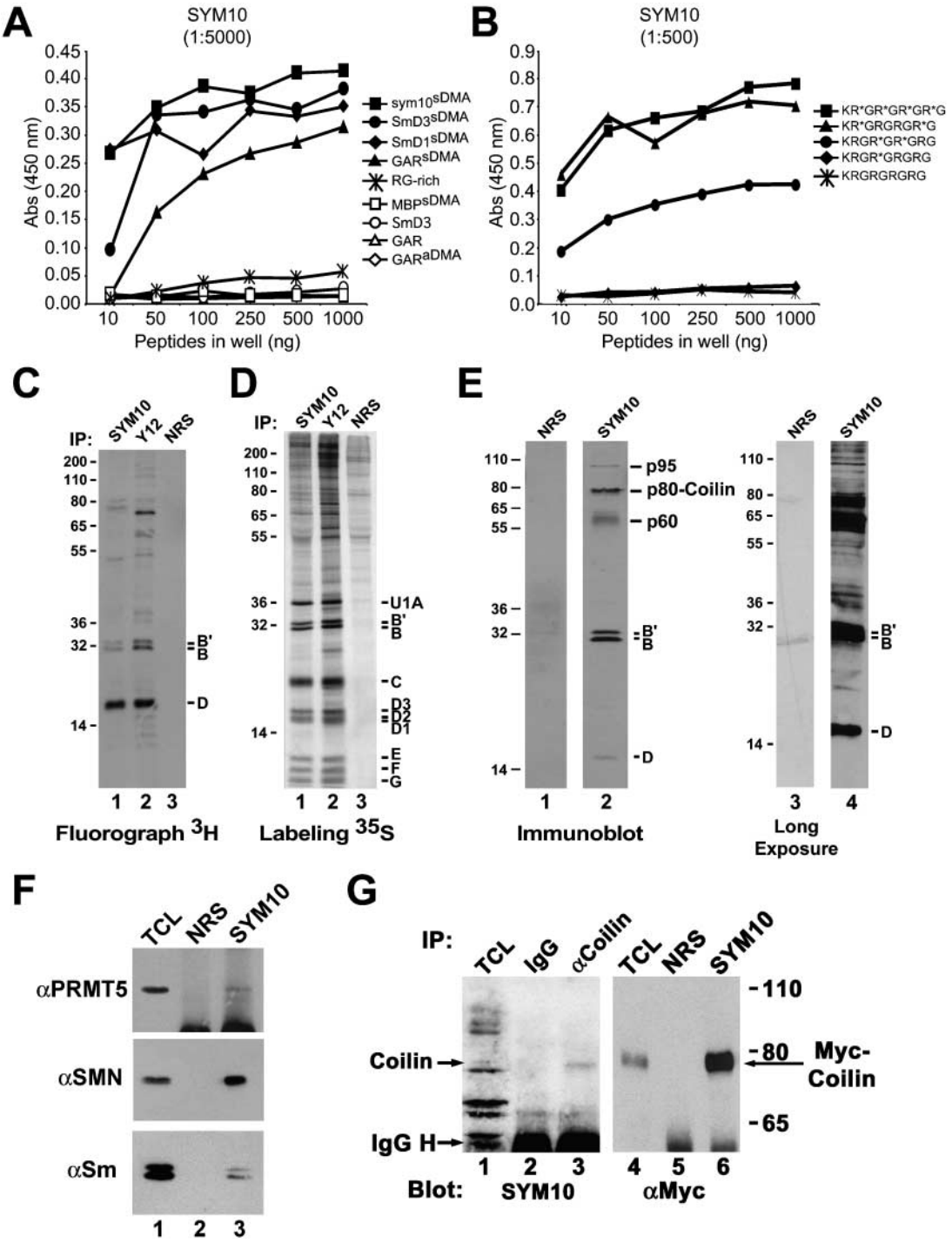

Figure 1. **SYM10 recognizes many cellular proteins including coilin and the Sm proteins B, B′, and D.** (A and B) The specificity of SYM10 was examined using an ELISA. The quantity of peptide is indicated on the abscissa and the absorbance on the ordinate. (C) HeLa cells were metabolically labeled with (*methyl*-^{3}H)-L-methionine in the presence of translation inhibitors, lysed, and immunoprecipitated with the indicated antibodies. The ^{3}H-labeled proteins were visualized by fluorography. The migration of SmB, B′, and D proteins is indicated. (D) HeLa cells were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with the SYM10 antibody, Y12, or normal rabbit serum. Proteins were separated using high TEMED SDS-PAGE. The ^{35}S-labeled proteins were visualized by autoradiography. The migration of Sm proteins and U1A is indicated. (E) HeLa cell lysates were immunoblotted with the NRS or the SYM10 antibody (1:1,000). (F) The methylosome and SMN complexes are immunoprecipitated by SYM10. Immunoprecipitation was performed in HeLa cells using SYM10, and the proteins were immunoblotted with PRMT5, SMN, and Sm B/B′ (ANA128) antibodies. (G) Coilin is recognized directly by SYM10. Immunoprecipitation was performed in HeLa cells using α-coilin antibodies followed by immunoblotting with SYM10 (1:1,000; left). Coilin is immunoprecipitated by SYM10. Immunoprecipitation was performed with the SYM10 antibody using HeLa cells transfected with myc-coilin followed by immunoblotting with α-Myc antibodies to detect transfected myc-coilin (right).

Table I. **The SYM10 antibody immunoprecipitates coilin**

Residues start–end	Mass observed	Mass expected	Sequence	Modifications
p80-coilin				
1–8	864.43	864.42	MAASETVR	
1–10	1133.55	1133.54	MAASETVRLR	
83–88	773.43	773.43	VKLEER	
132–136	713.36	713.35	HWKSR	
145–151	813.48	813.47	VLDLEPK	
182–186	615.36	615.35	RKSPK	
196–201	685.41	685.4	KAKNPK	
197–204	870.37	870.36	AKNPKSPK	
264–274	1233.62	1233.61	VTLEARNSSEK	
270–281	1332.61	1332.6	NSSEKLPTELSK	
275–281	787.48	787.47	LPTELSK	
288–297	1074.48	1074.52	NTTADKLAIK	
394–405	1509.68	1537.75	GWGREENLFSWK	2 methyls
394–408	1765.98	1791.69	GWGREENLFSWKGAK	2 methyls
406–413	876.3	848.45	GAKGRGMR	2 methyls + oxidation (M)
406–415	1074.52	1046.25	GAKGRGMRGR	2 methyls
406–415	1102.55	1046.25	GAKGRGMRGR	4 methyls
406–415	1089.53	1061.58	GAKGRGMRGR	2 methyls + oxidation (M)
406–417	1314.26	1258.7	GAKGRGMRGRGR	4 methyls
409–417	1030.44	1002.55	GRGMRGRGR	2 methyls
409–417	1058.42	1002.55	GRGMRGRGR	4 methyls
409–417	1046.39	1018.54	GRGMRGRGR	2 methyls + oxidation (M)
409–417	1074.52	1018.54	GRGMRGRGR	4 methyls + oxidation (M)
411–417	805.41	805.4	GMRGRGR	Oxidation (M)
411–417	861.28	805.42	GMRGRGR	4 methyls
411–419	1030.44	1002.55	GMRGRGRGR	2 methyls
411–419	1058.42	1002.55	GMRGRGRGR	4 methyls
411–419	1046.39	1018.54	GMRGRGRGR	2 methyls + oxidation (M)
411–419	1074.52	1018.54	GMRGRGRGR	4 methyls + oxidation (M)
430–435	720.38	720.38	STDNQR	

To identify proteins recognized by SYM10, a large scale immunoprecipitation was performed on HeLa cells followed by a MALDI-TOF mass spectrometry identification of the tryptic-digested proteins. The protein with an *M*r of 80 kD was identified as coilin (sequence data available from GenBank/EMBL/DDBJ under accession no. 4758024). The peptides derived from coilin were examined for the presence of DMA. Since methylation of arginines prevents cleavage by trypsin, peptide sizes that would result from the dimethylation of arginines in coilin were predicted. The table presents the residue numbers and the mass observed and expected of peptides found using the MALDI-TOF.

pected, SYM10 recognized a doublet of ~30 kD and a band of ~16 kD characteristic of Sm proteins B, B′, and D that were not detected with normal rabbit serum (Fig. 1 E). Major bands of apparent molecular weights of 95, 80, and 60 kD were also detected (Fig. 1 E). A longer exposure of a different SYM10 immunoblot demonstrated that many proteins contain sDMA (Fig. 1 E, lane 4). The fact that SYM10 can directly recognize SmB/B′ proteins in which sDMAs are found in a noncontiguous RG motif (Brahms et al., 2001) is in good agreement with the SYM10 epitope mapped by ELISA using synthetic peptides. The methylation of Sm B/B′ and D proteins is thought to occur in a complex dubbed the methylosome (Friesen et al., 2001b; Meister et al., 2001b). The presence of Sm proteins, SMN, and PRMT5 in the SYM10 complex was confirmed by probing SYM10 immunoprecipitates with their respective antisera (Fig. 1 F, lane 3). This shows that SYM10 can coimmunoprecipitate components of the methylosome and the SMN complexes.

To identify other proteins recognized by the SYM10 antibody, a large scale immunoprecipitation was performed on HeLa cells followed by a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry identification of the digested proteins. The protein with an apparent molecular weight of 80 kD was identified as coilin (gi:4758024; Table I). The proteins at 95 and 60 kD will be described elsewhere. Coilin contains multiple repeated RG motifs. The RG-rich regions of coilin are not known to be methylated but have been shown to be required for SMN interaction (Hebert et al., 2001). These RG-rich sequences are clustered in two repeated motifs that are both potential epitopes for SYM10. The peptides derived from coilin were examined for the presence of dimethylated arginines (Table I). Since methylation of arginines prevents cleavage by trypsin (Baldwin and Carnegie, 1971), we predicted peptide sizes that would result from the dimethylation of arginines in coilin. Peptide GWGR^{397}EENLFSWK contains two methyl groups, indicating arginine 397 is dimethylated (Table I). Peptide GAKGR^{410}GMR^{413}GR contains four methyls, indicating that both arginines 410 and 413 are dimethylated. The other peptide that is informative is GMR^{413}GR^{415}GR that contains four methyl groups, indicating that both arginines 413 and 415 are dimethylated. With the mass spectrom-

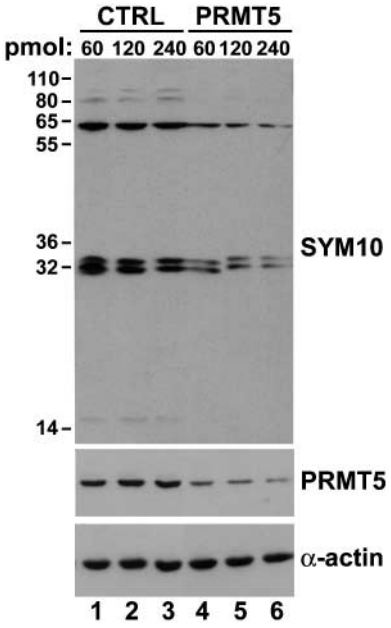

Figure 2. **Down-regulation of PRMT5 by siRNA results in a loss of recognized proteins by SYM10.** Control siRNAs (lane 1–3) or siRNAs engineered to specifically target PRMT5 mRNAs (lane 4–6) were introduced in Jurkat cells using electroporation. Cell extracts were prepared 72 h posttransfection, and the levels of SYM10 (top), PRMT5 (middle), and actin (bottom) were assessed by immunoblotting.

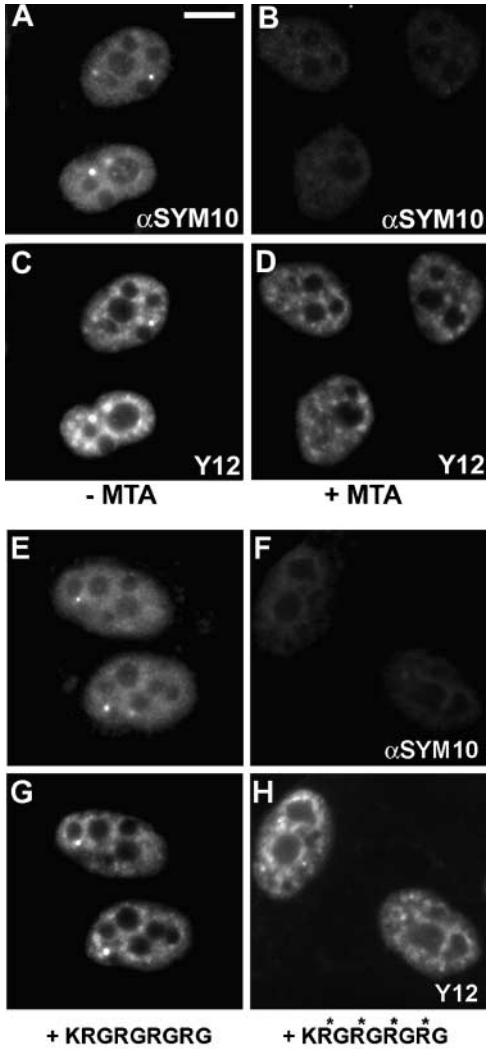

Figure 3. **Nuclear bright foci stained with SYM10.** HeLa cells treated with DMSO (−MTA) or the methylation inhibitor MTA (+MTA) for 24h were labeled for immunofluorescence using SYM10 (A and B) and the snRNP antibody Y12 (C and D). A peptide competition was performed using the immunizing peptide in a nonmethylated form (E and G) and in a fully methylated form (F and H). The antibody was preincubated with the peptide at a final concentration of 10 μM for 15 min on ice before immunofluorescence labeling using SYM10 (E and F) and the snRNP antibody Y12 (G and H). B and D, E and G, and F and H were double labeled, and the cells were visualized by fluorescence microscopy. Bar, 10 μM.

etry data, we can only assess the methylation of arginine 397, 410, 413, and 415 by using trypsin. But it is likely that arginines 417 and 419 are also dimethylated as this would provide epitopes for SYM10. Since the mass spectrometry identification does not distinguish between asymmetrical and symmetrical methylation, anticoilin immunoprecipitations were immunoblotted with the sDMA-specific SYM10 antibody (Fig. 1 G, left). A single band of ~80 kD could be detected by SYM10 in the coilin immunoprecipitate (Fig. 1 G, lane 3), indicating that coilin can indeed be recognized directly by SYM10 and thus contains sDMA. Finally, to confirm the mass spectrometry identification the reciprocal coimmunoprecipitation experiment was performed on myc-coilin–transfected cells (Fig. 1 G, right). As expected, myc-coilin was observed in SYM10 immunoprecipitates (Fig. 1 G, lane 6). Together, these results demonstrate that coilin is an sDMA-containing protein in vivo.

PRMT5 is the only methyltransferase known to generate sDMAs (Branscombe et al., 2001), and we have shown that it is present in SYM10 immunoprecipitates (Fig. 1 F). It is thus inferred that PRMT5 would be the enzyme responsible for producing the SYM10 epitopes. To verify this hypothesis, we made use of a small interfering RNA (siRNA) approach to knock-down PRMT5 expression in vivo. A control siRNA or an siRNA engineered to specifically target PRMT5 mRNA was introduced in Jurkat cells using electroporation. Cell extracts were prepared 72 h posttransfection, and the levels of PRMT5 protein was assessed by immunoblotting (Fig. 2). Introducing 60 to 240 pmoles of control siRNA had no effect on PRMT5 protein levels (Fig. 2, middle, lanes 1–3). In contrast, the same amounts of siRNA directed against PRMT5 produced a gradual decrease in PRMT5 protein level (Fig. 2, middle, lanes 4–6). Strikingly, a similar decrease is observed in the SYM10 immunoblot signal (Fig. 2, top, lanes 4–6). Equivalent amounts of total proteins was present in each lane as assessed by antiactin immunoblotting (Fig. 2, bottom). These results suggest that PRMT5 is one of the enzymes responsible for generating SYM10 epitopes, including the ones found in Sm proteins B/B′ and D, coilin, p60, and p95.

sDMA-containing proteins are nuclear and concentrated in nuclear foci

The cellular distribution of sDMA-containing proteins was examined by indirect immunofluorescence using the SYM10 antibody in HeLa cells. SYM10 stained bright foci and the

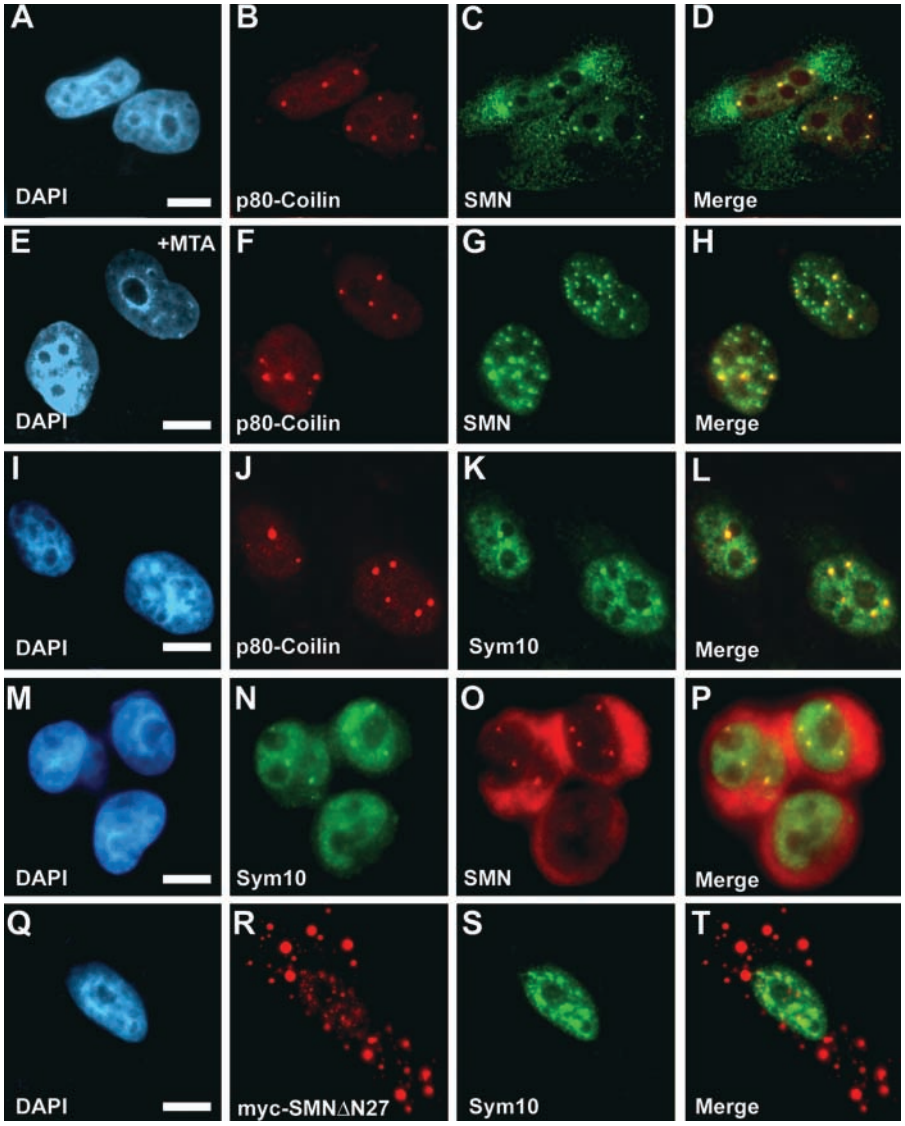

Figure 4. **SMN colocalizes with sDMA-containing proteins in Cajal bodies.** HeLa cells were labeled for immunofluorescence with a coilin antibody (B) and a SMN antibody (C). Colocalization within Cajal bodies of both proteins is shown in the merged image (D). HeLa cells treated with MTA (+MTA) for 24 h were immunostained for coilin (F) and SMN (G). HeLa cells were labeled for immunofluorescence with a coilin mAb (J) and with the SYM10 antibody (K). HeLa cells were labeled for immunofluorescence with a SMN antibody (O) and with SYM10 (N). HeLa cells were transfected with myc-SMNΔN27 and immunostained using the SYM10 antibody (S) and a myc antibody (R). The merged image is shown in T. Cell nuclei were counterstained with DAPI (A, E, I, M, and Q, blue). Bars, 10 μM.

nucleoplasm but not the nucleolus nor the cytoplasm (Fig. 3 A). To demonstrate the specificity of our antibody for methylated proteins, we treated HeLa cells with the methylation inhibitor 5′-deoxy-5′-methylthioadenosine (MTA) for 24 h. The absence of a signal with the SYM10 antibody after MTA treatment confirmed that SYM10 was methyl specific (Fig. 3 B). The anti-snRNP antibody Y12 has also been reported to recognize sDMA-containing Sm proteins (Brahms et al., 2000). To examine whether the SYM10 antibody staining resembled the snRNPs antibody Y12 staining in HeLa cells, immunofluorescence was performed on HeLa cells untreated or treated with MTA. The anti-snRNP antibody Y12 stained predominantly IGCs, and the signal did not change after MTA treatment (Fig. 3, C and D). These observations demonstrate that SYM10 is methyl specific and that, at least in situ, the anti-snRNP antibody Y12 recognizes other nonmethylated epitopes in snRNPs. To confirm the specificity for sDMA-containing proteins in these experiments, immunofluorescence was performed in the presence of saturating amounts of the methylated sym10 peptide. Again, only the SYM10 signal was affected by preincubation with the sDMA-containing sym10 peptide (Fig. 3, F and H). Preincubation with the unmodified derivative of the sym10 peptide had no effect on the immunofluorescence signal of either SYM10 or Y12 (Fig. 3, E and G). These findings demonstrate that proteins containing sDMA are predominantly nuclear and concentrated in bright foci in HeLa cells.

Proteins containing sDMA colocalize with coilin in Cajal bodies

To demonstrate that coilin and SMN colocalize in the cells used in this study, T4 HeLa cells were immunostained using a coilin rabbit polyclonal antibody (Andrade et al., 1993) (Fig. 4 B) and an anti-SMN mAb (Fig. 4 C). The merged images demonstrate a perfect colocalization within Cajal bodies (Fig. 4 D). These findings demonstrate that SMN and coilin colocalize in T4 HeLa cells. Cells were treated with MTA to examine the effect on SMN and coilin localization. SMN concentrated in a drastically increased number of bright foci even though the protein levels of SMN did not change (Fig. 4 G; unpublished data), whereas the localization of coilin remained unchanged (Fig. 4 F) and did not accumulate in the SMN bright foci (Fig. 4 H). Interestingly

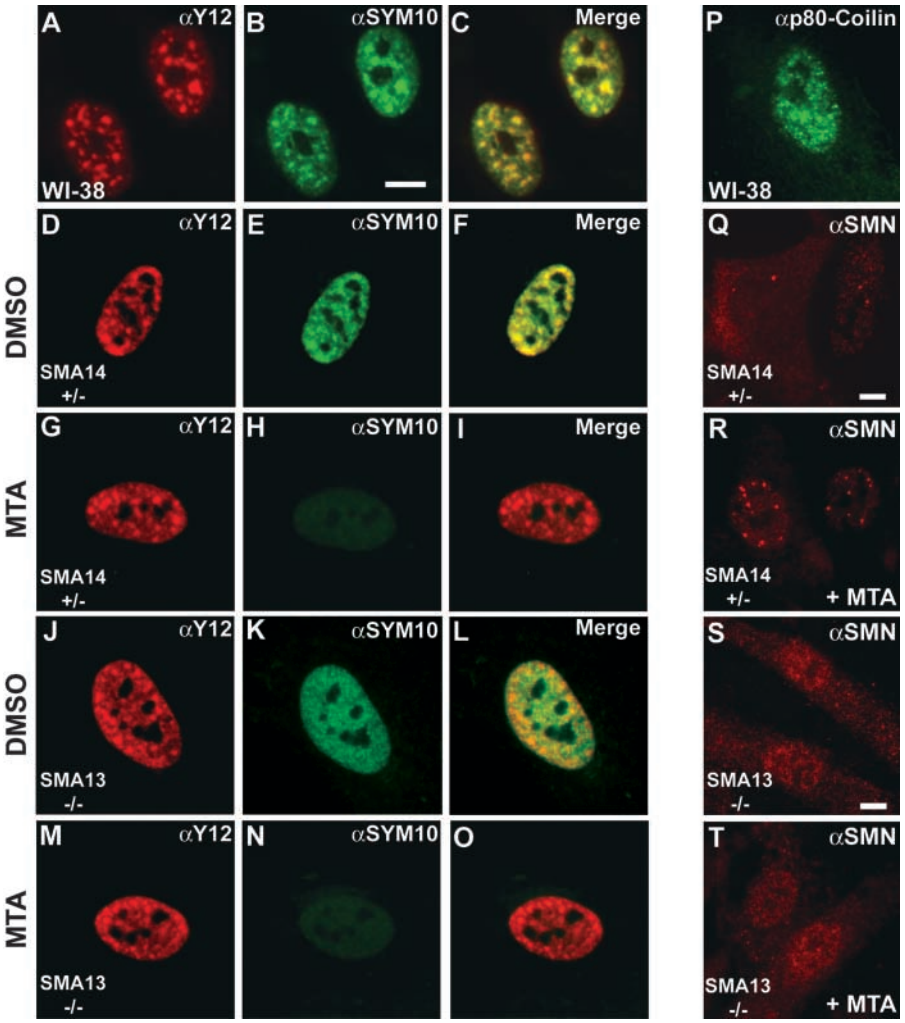

Figure 5. **The localization of sDMA proteins is disrupted in a patient with SMA.** Human fibroblasts WI-38 were doubly stained with Y12 (A) and SYM10 (B) or an anticoilin antibody (P). Fibroblasts from a patient with Werdnig-Hoffman disease SMA type I, *SMN1*−/−, GM03813 (SMA13), and fibroblasts from his unaffected mother *SMN1*−/+ GM03814 (SMA14) were either mock treated (DMSO, D–F and J–L) or treated with 750 μM MTA (G–I and M–O) for 24 h and stained using Y12 (D, G, J and M) and SYM10 (E, H, K and N). Fibroblasts from the patient with SMA (SMA13) or his unaffected mother (SMA14) were treated with DMSO (Q and S) or treated with 750 μM MTA (MTA, R and T) for 24 h and stained using anti-SMN antibodies (Q–S). Bars, 5 μm.

this relocalization of SMN was not observed with another commonly used methylation inhibitor adenosine-2',3′-dialdehyde (Adox; unpublished data). Upon MTA treatment, coilin colocalized with SMN in Cajal bodies, but the relocalized SMN accumulated in gems (Fig. 4 H). The change in SMN localization is most likely due to the lack of binding partners for SMN.

To confirm that the nuclear foci recognized by SYM10 are Cajal bodies, HeLa cells were costained by using both SYM10 and anticoilin antibodies. The bright foci stained by SYM10 colocalized with Cajal bodies as observed with an anticoilin mAb (Almeida et al., 1998) (Fig. 4, I–L). To determine whether these bright foci also colocalized with SMN, HeLa cells were costained using both SYM10 (Fig. 4 N) and an anti-SMN antibody (Fig. 4 O). The nuclear bodies stained with the SYM10 antibody coincide exactly with the ones stained with the anti-SMN antibody as shown in the merged images (Fig. 4 P). To determine whether SMN influences the localization of sDMA-containing proteins, a dominant-negative mutant of SMN lacking the first 27 amino acids at the NH_2 termini (SMNΔN27 [Pellizzoni et al., 1998]) was transfected in HeLa cells. Expression of SMNΔN27 in HeLa cells resulted in the apparition of enlarged cytoplasmic and nuclear structures (Fig. 4 R) as described previously (Pellizzoni et al., 1998). Interestingly, the nuclear distribution of sDMA-containing proteins was altered in cells transfected with SMNΔN27 to the enlarged nuclear structures as visualized by using SYM10 (Fig. 4 S). The nuclear staining observed with SYM10 is consistent with coilin being a major epitope in HeLa cells because coilin has been shown to relocalize with SMNΔN27 (Pellizzoni et al., 1998).

sDMA-containing proteins localize to IGCs in cells containing low levels of SMN

Primary fibroblasts are essentially devoid of Cajal bodies, and coilin is primarily localized diffusely in the nucleoplasm in these cells (Fig. 5 P) (Spector et al., 1992; Carmo-Fonseca et al., 1993). To determine the localization of sDMA-containing proteins in cells lacking Cajal bodies, immunofluorescence was performed on WI-38 cells using the snRNPs antibody Y12 (Fig. 5 A) and the SYM10 antibody (Fig. 5 B). Both antibodies costained a pattern that was characteristic of IGCs (Fig. 5 C). Patients with SMA have less SMN protein, and the severity of the disease inversely correlates with the number of gems (Coovert et al., 1997). The cellular distribution of sDMA-containing proteins was compared in WI-38 cells and fibroblasts from a patient with SMA and his unaffected mother. The unaffected mother derived fibroblast cells had Sm proteins (Fig. 5 D) and sDMA-containing pro-

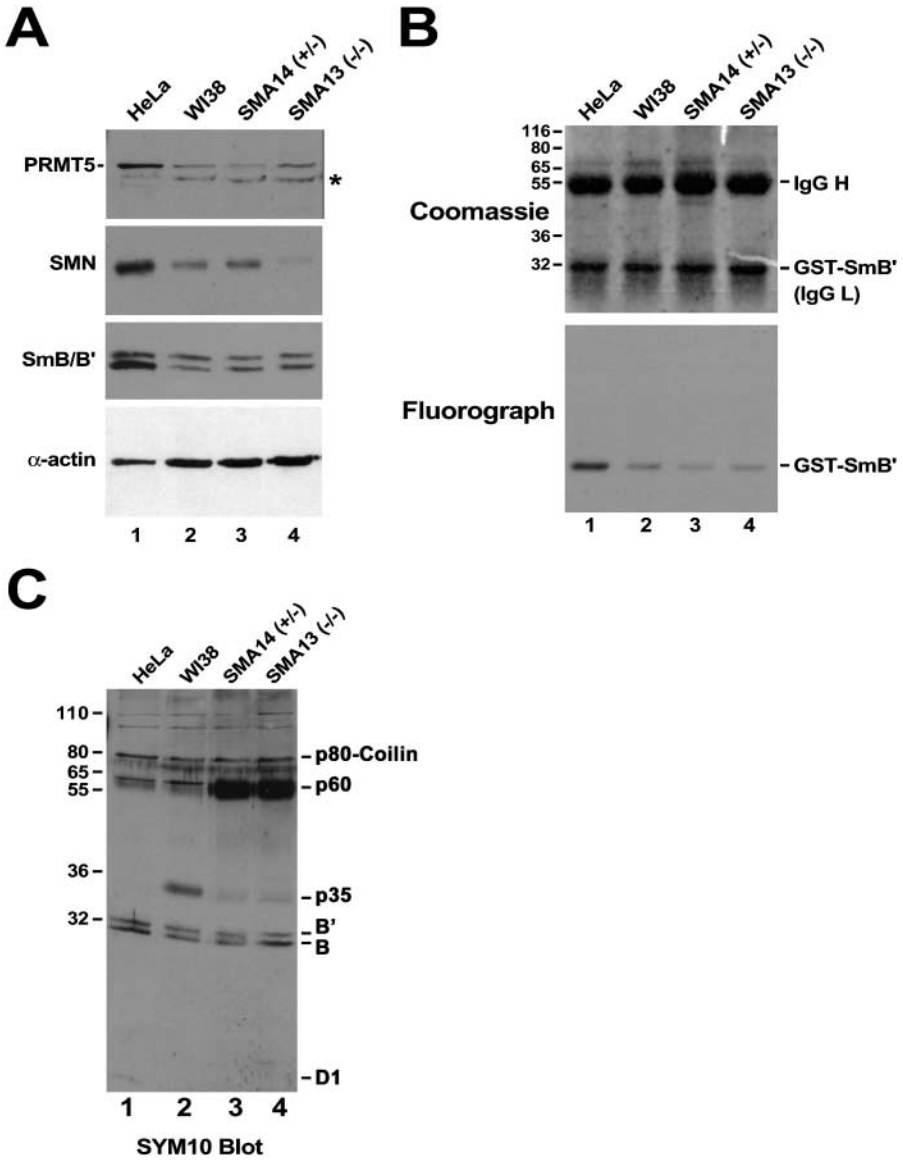

Figure 6. **The level of PRMT5 and sDMA-containing proteins is not affected in the SMA cell line.** (A) Cell lysates from the indicated cell lines were immunoblotted with PRMT5, SMN, SmB/B′ (ANA128), or actin antibodies. The asterisk indicates a nonspecific band. (B) PRMT5 immunoprecipitations were performed using the indicated cell lines. Immunoprecipitated proteins were incubated with GST–SmB′ as an exogenous substrate in the presence of (methyl-^{3}H)–SAM. Proteins were resolved by SDS-PAGE, stained with Coomassie blue (top), and visualized by fluorography (bottom). The migration of GST–SmB′ IgG H and L chains are indicated on the right. (C) SYM10 immunoblotting was performed on extracts prepared from the indicated cell lines.

teins (Fig. 5 E) colocalizing in IGCs (Fig. 5 F), similar to the primary fibroblasts WI-38 (Fig. 5, A–C). The cells derived from the patient with SMA contained Sm proteins localized within IGCs as observed by using the snRNP antibody Y12 (Fig. 5 J). However, the localization of sDMA-containing proteins was distinct: sDMA-containing proteins were distributed in the nucleoplasm as a discrete granular pattern (Fig. 5 K). Treatment of cells with the methyltransferase inhibitor MTA had no effect on the localization of Sm proteins in snRNPs recognized by the antibody Y12 (Fig. 5, G and M), but the fluorescence signal for SYM10 was reduced considerably (Fig. 5, H and N). The SMN localization was examined in cells derived from the SMA patient. The unaffected mother derived cells concentrated SMN in gems which increased in number after MTA treatment (Fig. 5, Q compared with R). The SMA patient derived cells did not concentrate SMN in gems (Fig. 5 S), and treatment of cells with MTA had no effect on the localization of SMN (Fig. 5 T). These observations suggest that patients with SMA mislocalize sDMA-containing proteins.

The level of PRMT5 and sDMA-containing proteins is not affected in the SMA cell line

To investigate the levels of PRMT5 protein levels and activity, lysates from HeLa, WI-38, and SMA cell lines (mother and affected child) were immunoblotted with an anti-PRMT5 antibody. HeLa cells contained sixfold higher protein levels of PRMT5 than the three fibroblasts cell lines examined when normalized to actin by densitometric analysis (Fig. 6 A, lanes 1–4). SMN was elevated in HeLa cells, intermediate in WI-38 and the unaffected SMA mother, and low in the patient with SMA (Fig. 6 A). HeLa cells contained more total SmB/B′ proteins than fibroblasts, but no differences were observed between normal and affected SMA cell lines when normalized to actin (Fig. 6 A, lanes 1–4). Anti-PRMT5 immunoprecipitates followed by in vitro methylation assays using (methyl-^{3}H) S-adenosyl-L-methionine and GST-SmB′ as an exogenous substrate were performed to assess the levels of PRMT5 activity between the cell lines. The proteins from the in vitro reactions were resolved by SDS-PAGE, stained with Coomassie blue (Fig. 6 B, top), and the methylated proteins were visualized by

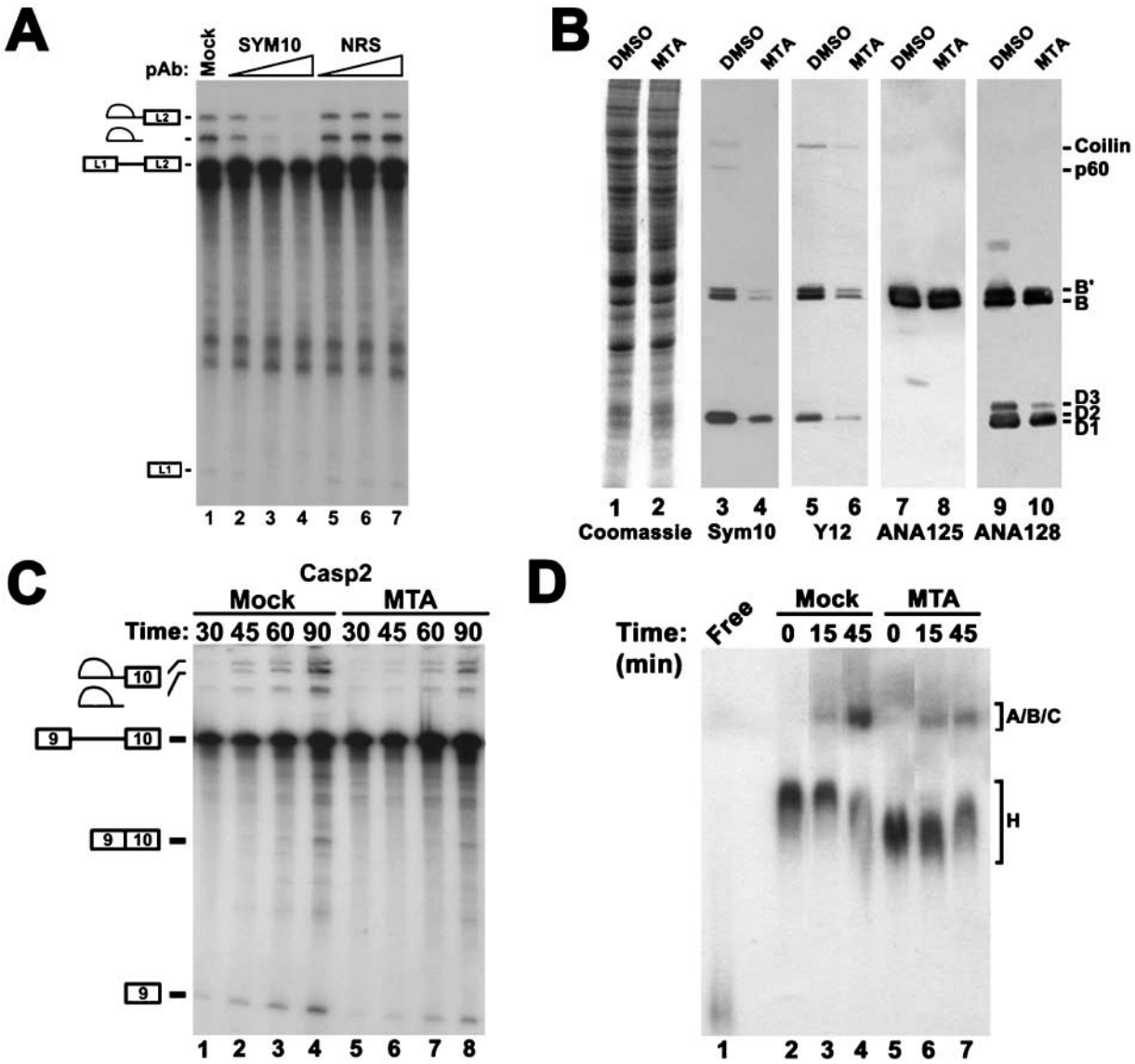

Figure 7. **Pre-mRNA splicing and spliceosomal formation is impaired in hypomethylated nuclear extracts, and SYM10 inhibits pre-mRNA splicing.** (A) Splicing reactions were performed by using a ^{32}P-labeled AdML transcription unit pre-mRNA substrate (2 fmoles) in the presence of increasing amount (25–250 ng) of purified antibodies. RNAs were resolved on an 8% denaturing polyacrylamide gel. The positions of pre-mRNA and splicing products and intermediates are indicated on the left. (B) DMSO (Mock) or MTA-treated extracts were stained with Coomassie blue or immunoblotted with the indicated antibodies. (C) Splicing reactions were performed by using a ^{32}P-labeled caspase 2 pre-mRNA. The pre-mRNA transcripts were incubated with either the mock-treated or with the MTA-treated nuclear extracts for increasing amount of time. RNAs were resolved on a 6% denaturing polyacrylamide gel. (D) Splicing complexes assembly on the ^{32}P-labeled adenovirus major late transcripts using nuclear extracts that are either mock treated or MTA treated. Heterogeneous (H) and spliceosomal complexes (A/B/C) are indicated on the left.

fluorography (Fig. 6 B, bottom). HeLa cells contained higher levels of PRMT5 activity than the fibroblasts cell lines (Fig. 6 B, lanes 1–4) consistent with higher levels of PRMT5 protein. However, the levels of sDMA-containing proteins was not higher in HeLa cells compared with the fibroblasts by using SYM10 to immunoblot whole cell extracts (Fig. 6 C). Comparable levels of methylated coilin were also observed between the four cell lines (Fig. 6 C, lanes 1–4, see indicated band). However, qualitative and quantitative differences were observed for some unidentified methylated proteins recognized by SYM10, including p60 (Fig. 6 C, lanes 1–4, see indicated bands). Nonetheless, no methylation differences were observed between the mother and the SMA patient (Fig. 6 C, lanes 3 and 4). These results demonstrate that the distinct localization of sDMA-containing proteins in SMA patients is not related to the reduced expression and/or activity of PRMT5 in these cells. Our results suggest that it is the SMN protein levels that correlates well with the observed differences in cellular distribution of sDMA-containing polypeptides.

The sDMA-specific antibody SYM10 inhibits pre-mRNA splicing

To confirm whether sDMA methylated proteins are required for splicing in vitro, nuclear extracts were preincu-

bated with an increasing amount of affinity-purified SYM10 antibody. The splicing of radiolabeled adenovirus major late (AdML) transcripts was assessed (after 2 h), and splicing products were analyzed by denaturing gel electrophoresis. A complete inhibition of pre-mRNA splicing was achieved by the addition of increasing amounts of the SYM10 antibody (Fig. 7 A, lanes 2–4) but not by normal rabbit serum (Fig. 7 A, lanes 5–7). Preincubation of SYM10 with the methylated sym10 peptide prevented the inhibition of splicing, showing the effect is specific (unpublished data). The inhibition of splicing by SYM10 resembles the inhibition observed with Y12 (Padgett et al., 1983). However, our immunofluorescence results show that Y12 likely recognizes other nonmethylated epitopes. The inhibition observed here with the SYM10 antibody demonstrates that sDMA-containing proteins are part of the active spliceosome.

Pre-mRNA splicing and spliceosomal complex formation are impaired in hypomethylated nuclear extracts

To further confirm whether arginine methylation is a necessary modification for pre-mRNA splicing, nuclear extracts were prepared from HeLa cells grown in the presence of the methylation inhibitor MTA. The methylation status of cellular proteins was assessed by using the SYM10 antibody. More than 90% of the arginine methylation of SmB/B′, coilin, and p60 and 75% of the methylation of SmD1 proteins was lost after treatment as determined by densitometry (Fig. 7 B, lanes 3–4). There was no significant difference in the level of total proteins (Fig. 7 B, lanes 1–2) and the level of SmB and SmD proteins in the nuclear extract of mock-treated and MTA-treated cells (Fig. 7 B, lanes 7–10). Although the immunofluorescent signal with the anti-Y12 antibody did not diminish with MTA (Fig. 3), a reduction in the level of SmB, B′, D, and coilin was observed in MTA-treated extracts when immunoblotted with Y12 (Fig. 7 B, lanes 5 and 6). These findings confirm that Y12 recognizes methylated Sm proteins (Brahms et al., 2000) and a methylated protein of 80 kD that has been identified recently as coilin (Hebert et al., 2002).

The splicing of a caspase-2 pre-mRNA substrate (Côté et al., 2001) was assayed in nuclear extracts prepared from mock-treated (Fig. 7 C, lanes 1–4) or MTA-treated HeLa cells (Fig. 7 C, lanes 5–8). Overall splicing efficiency was reduced twofold in hypomethylated extracts and was most noticeable by a slower rate of apparition of splicing intermediates: e.g., note the amount of lariat intermediates in the mock-treated extract after 45 min incubation (Fig. 7 C, lane 2 compared with 6). A similar inhibition was observed when using the AdML splicing substrate (unpublished data). Splicing by hypomethylated extracts was still sensitive to inhibition by the SYM10 antibody, indicating that the residual splicing activity is due to the small proportion of methylated proteins remaining in the extract (unpublished data). The formation of splicing complexes on the AdML transcripts was assayed using aliquots of a splicing reaction performed in mock- or MTA-treated nuclear extracts (Fig. 7 D). After 0, 15, and 45 min of incubation, complexes were resolved by using native gels. Mock-treated nuclear extracts supported the formation of normal spliceosomal complexes (A, B, and C) and heterogeneous (H) complexes (Fig. 7 D, lanes 2–4 [Konarska and Sharp, 1986]). However, hypomethylated nuclear extracts showed less conversion into spliceosomal complexes after a 45-min incubation (Fig. 7 D, lanes 5–7). Moreover, the loading of hnRNPs onto the pre-mRNA (complex H) was also affected as it migrated slightly faster, suggesting an aberrant composition. This is consistent with the fact that hnRNPs are a major class of proteins modified by arginine methylation (Liu and Dreyfuss, 1995). These studies suggest that normal levels of sDMA-containing proteins are required to support efficient pre-mRNA splicing.

Discussion

By using a new sDMA-specific antibody (SYM10), we identified coilin as an sDMA-containing protein. Immunoprecipitated coilin was recognized directly by SYM10 immunoblotting, and the in vivo methylation of coilin was further demonstrated by MALDI-TOF mass spectrometry. Immunofluorescence studies demonstrated that sDMA-containing proteins are localized diffusely in the nucleoplasm and concentrated in Cajal bodies in HeLa cells. Gems were not observed in the untreated T4 HeLa cells used: coilin and SMN colocalized perfectly in Cajal bodies (Fig. 4). In the presence of the methylase inhibitor MTA, the appearance of dozens of gems was observed. Coilin methylation was decreased with MTA treatment, suggesting that its methylation is necessary to maintain SMN in the Cajal bodies. Primary fibroblasts that are devoid of Cajal bodies concentrated sDMA-containing proteins in IGCs or speckles. Cells derived from a patient with SMA localized its sDMA-containing proteins in the nucleoplasm as a discrete granular pattern. SMN may play a role in localizing sDMA-containing proteins because the expression of a dominant-negative SMN (SMNΔN27) concentrated sDMA-containing proteins in enlarged nuclear structures rather than Cajal bodies. Moreover, our findings show that splicing reactions were efficiently inhibited in the presence of SYM10 antibody and by using hypomethylated nuclear extracts.

SYM10, a symmetrical dimethylarginine-specific antibody

The presence of antibodies that recognize posttranslational modifications has greatly enhanced our ability to understand these modifications in vivo (e.g., phosphorylation). The field of arginine methylation lacks antibodies that can recognize sDMA-containing polypeptides. The antibody we generated, SYM10, recognizes sDMA and not aDMA-modified proteins containing GAR sequences. More specifically, we have defined that SYM10 requires at least two preferentially spaced sDMA-G residues for reactivity. This is consistent with the fact that SYM10 recognized SmB/B′ (R^{sDMA}GGPPPPMGR^{sDMA}G) and SmD1 (9 R^{sDMA}G repeats), which are known to contain sDMA in vivo. Furthermore, we show that the SYM10 epitopes are generated by PRMT5 (Fig. 2), which is the enzyme thought to methylate symmetrically Sm proteins (Friesen et al., 2001b; Meister et al., 2001b). The complete loss of immunostaining with MTA treatment further demonstrates that SYM10 also recognizes methylated epitopes in cells. In contrast, the immunostaining of IGCs is

not lost by using the anti-snRNP antibody Y12. However, immunoblotting with Y12 demonstrated that the SmB and D epitopes were reduced after MTA treatment, demonstrating that it indeed does recognize methylated epitopes as reported previously (Brahms et al., 2000). The recognition of many proteins by SYM10 immunoblotting demonstrates that there exist a plethora of proteins with sDMA.

The Cajal body contains methylated coilin

We show that human coilin arginines 397, 410, 413, and 415 are dimethylated in vivo. These arginines are localized in GAR regions, which are well known sites of arginine methylation (Gary and Clarke, 1998). The methylase that catalyzes this posttranslational modification is unknown, but is likely to be the PRMT5 methylosome, since the arginines are located within a consensus sequence for PRMT5 (Friesen et al., 2001b; Meister et al., 2001b). The methylation of coilin may be a signal to rapidly target it to SMN complexes or vice versa and cause an accumulation in Cajal bodies (Hebert et al., 2001, 2002). The addition of MTA but not Adox (unpublished data) relocalized SMN exclusively in gems. It is not known why MTA is the only methylation inhibitor that causes this redistribution. However, our observations suggest that methylation regulates the components of Cajal bodies and regulates the appearance of gems. Thus, the presence of gems may be a marker for methylation activity in cells. Our data provide a function for coilin in the recruitment of Cajal body components. It is not known whether arginine methylation is regulated, but coilin may be a protein "sensor" for the levels of sDMA-containing snRNPs. Thus, it would be the levels of Sm proteins that would ultimately regulate Cajal body formation as suggested by Sleeman et al. (2001). Thus high levels of methylated snRNPs such as in rapidly dividing cells (HeLa) may increase the levels of methylated coilin, resulting in the recruitment of SMN complexes to the Cajal body for snRNP biogenesis and recycling.

The observation that SYM10 detects proteins within the nucleus and not the cytoplasm suggest that sDMA is a signal for nuclear import and that the nuclear import machinery is tightly coupled to the sDMA-generating methylosome(s) as proposed by Friesen et al. (2001b) and Meister et al. (2001b). In agreement with this model, SMN was found recently to be part of a preimport complex containing importin β, Snurportin, and ZPR1 (Gangwani et al., 2001; Narayanan et al., 2002), which suggests that SMN may accompany newly assembled snRNPs to the nucleus. The accumulation of SMN in MTA-treated cells is consistent with the model that SMN functions in snRNP biogenesis or recycling within the nucleus (Pellizzoni et al., 1998). Thus, in the absence of methylated snRNPs there would be no need to have SMN in Cajal bodies for snRNP biogenesis, and SMN would accumulate in a nuclear body, the gem, which is devoid of snRNPs. Therefore, the presence of gems may be a reflection of the general absence of methylated proteins.

In this study, three cell types expressing different levels of SMN protein were used to examine the cellular distribution of sDMA-containing proteins. The only difference observed between the cell types was the levels of SMN protein as reported previously (Gangwani et al., 2001). The levels of SmB protein have been shown to be lower in primary human fibroblast and may account for the absence of Cajal bodies in these cells (Sleeman et al., 2001). Indeed, the overexpression of SmB caused the appearance of Cajal bodies (Sleeman et al., 2001). In our studies, the levels of SmB and B′ were not significantly lower in the human fibroblasts compared with HeLa cells. The activity of PRMT5 was higher in HeLa cells, but this did not appear to affect the levels of methylated proteins as detected by SYM10 immunoblotting. In HeLa cells, the highest SMN-expressing cell line, sDMA-containing proteins were detected in Cajal bodies and not in IGCs. It is unknown why the IGCs were not detected, especially since SmB and D proteins are major epitopes by immunoblotting. A possibility is that methylated epitopes may be blocked by the presence of SMN that binds methylated proteins (Friesen et al., 2001a). Thus, the diffuse nuclear SMN complexes may mask the methylated proteins in IGCs in HeLa cells. Alternatively SMN may be involved in the nuclear organization of sDMA-containing proteins. The argument for this is that the sDMA-containing proteins of the unaffected mother and the SMA patient were different. Also consistent with this is the fact that SMNΔN27 reorganizes sDMA-containing proteins.

Methylated proteins and SMA

Treatment of the cells derived from the SMA patient with MTA did not induce gem formation. These data suggest that the little quantity of SMN that is present in these cells is not sufficient to concentrate in nuclear foci as detected by immunofluorescence. Thus, by having lower levels of SMN the cells have indirectly lost the ability to respond to changes in the methylation status of their proteins. The presence of mutations in *SMN1* that disrupt association with its RG-rich substrates (Pellizzoni et al., 1999) suggest that these are loss of function mutations and would be predicted to also have mislocalized sDMA-containing proteins. The observation that the anti-snRNP antibody Y12 stained IGCs in the SMA patient cells confirmed that the overall organization of snRNPs is not perturbed. It is interesting that the discrete granular pattern observed resembles sites of transcription (Fakan and Nobis, 1978). These findings show that the lower level of SMN in patients with SMA has a major effect on the nuclear distribution of methylated proteins.

Arginine methylation and pre-mRNA splicing

Our data suggest that the spliceosomal complexes do not assemble properly and may be aberrant in hypomethylated nuclear extracts. The two major known components that would be affected by using methylation inhibitors include the hnRNPs and the Sm proteins in snRNPs. The aberrant migration of the H complex observed is consistent with the observation that hnRNPs are a major family of proteins modified by arginine methylation (Liu and Dreyfuss, 1995). Thus methylation may affect their RNA binding activity and nucleocytoplasmic shuttling capabilities (McBride and Silver, 2001). The absence of methylation most likely prevents the assembly of snRNPs. Moreover, the absence of methylation would also prevent the methylation of the COOH-terminal regions of Sm proteins B and D and may prevent these proteins from making direct RNA contacts

with the pre-mRNAs. This is consistent with genetic studies performed in yeast, demonstrating that the COOH-terminal regions of the Sm proteins B, D1, and D3 are critical for pre-mRNA splicing and cell viability (Zhang et al., 2001). However, it is likely that there are other protein components of the spliceosome that are methylated and this will require further characterization.

In conclusion, our data show that the methylation of coilin causes SMN to localize in Cajal bodies. In hypomethylated cells, SMN localizes in gems. These findings demonstrate that arginine methylation regulates gem formation and is essential to maintain the integrity of the Cajal body. In addition, our data show that a patient with SMA is unable to properly localize its sDMA-containing proteins. We also show that pre-mRNA splicing reactions require the presence of sDMA for function.

Materials and methods

Antibodies

SDMA, ADMA, and nonmethylated arginines containing peptides were synthesized at W.M. Keck Biotech Resource Center. Polyclonal antibodies were generated by using New Zealand rabbits injected with peptides coupled to keyhole limpet hemocyanin (Sigma-Aldrich). The peptides used for anti-PRMT5 and anti-SYM10 antibodies were KNRPGPQTRSDLLLSGRDWN and KR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}G. SYM10 was affinity purified (UBI, Upstate). The HA antibody is 12CA5 (Babco). The 9E10 myc antibody was from the American Type Culture Collection. The SMN antibody is from Transduction Laboratories. The Y12 hybridoma was provided by Robin Reed (Harvard University, Boston, MA). The SmB antibody (ANA125) and SmB and D antibody (ANA128) are from Cappel. The antibody against α-actin is from Sigma-Aldrich. The coilin antibodies were gifts from Drs. Chan (Scripps Research Institute, La Jolla, CA) and Matera (Case Western Reserve University, Cleveland, OH).

ELISA

ELISA plates (Costar) were coated with the indicated peptides and quantitated by using spectrophotometry. The following peptides were used: sym10^{sDMA} (KR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}G), SmD3^{sDMA} (KAAILKAQVAAR^{sDMA}GR^{sDMA}GR^{sDMA}GMGR^{sDMA}G), SmD1^{sDMA} (SRRASVAGR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}GG), GAR^{sDMA} (KFGGR^{sDMA}GGGR^{sDMA}GGGR^{sDMA}GGFGGR^{sDMA}GGR^{sDMA}GG), RG-rich (KGRGRGRGPPPPPRGRGRGRG), MBP^{sDMA} (SRRASVPSQGKGR^{sDMA}GLSLSR), SmD3 (KAAILKAQVAARGRGRGMGRG), GAR (KFGGR^{sDMA}GGGR^{sDMA}GGGR^{sDMA}GGFGGR^{sDMA}GGR^{sDMA}GG), and GAR^{aDMA} (KFGGR^{aDMA}GGGR^{aDMA}GGGR^{aDMA}GGFGGR^{aDMA}GGR^{aDMA}GG).

DNA constructs

Myc-coilin in pSG5 (Bohmann et al., 1995) was obtained from G. Matera. SMNΔN27 was obtained by RT-PCR using HeLa cells RNA.

SiRNA knock-downs

SiRNAs were obtained from Dharmacon Research. PRMT5 siRNA was derived from the PRMT5 sequence (XM_033433) nucleotides 1,598–1,620. Control siRNA was the Luciferase GL2 duplex (no. D-1120–05; Dharmacon Research). Cells were washed twice in RMPI + 1% Hepes and diluted to 10^6 cells/ml. 0.5 ml was transferred into 0.4-mm gap electroporation cuvettes (Bio-Rad Laboratories) along with 6 μg of carrier DNA and 60, 120, or 240 pmoles of the respective siRNA. Electroporation was 280 V and 950 μF. Cells were then incubated at RT for 15 min and replated in complete media. Cells were lysed after 72 h.

Immunofluorescence and protein expression

T4 HeLa cells were cultured directly on coverslips into a 6-well dish (Maddon et al., 1986). For drug treatment, cells were incubated for 24 h with the vehicle (DMSO) or with the methyltransferase inhibitor MTA (Sigma-Aldrich) at a final concentration of 750 μM. Transfection of HeLa cells for immunofluorescence was achieved using Lipofectamine Plus. Cells were fixed with 1% paraformaldehyde in 1× PBS at pH 7.4 and permeabilized with 0.5% Triton X-100 in PBS. The cells were visualized with a Axioplan fluorescence microscope (Carl Zeiss MicroImaging, Inc.). Immunoprecipitations were performed using 1 μg of the respective antibody in a 1% Triton X-100 lysis buffer as described previously (Bedford et al., 2000).

Mass spectrometry

sDMA-containing proteins were immunopurified from 5×10^8 HeLa cells using 1 mg of polyclonal SYM10 antibody coupled to 1 g of protein A–Sepharose (Sigma-Aldrich). After washes, bound proteins were eluted with the SYM10 peptide. Proteins were resolved by SDS-PAGE and revealed by Coomassie blue staining. The protein bands were excised, in-gel digested with trypsin, and analyzed by MALDI-TOF on a Voyager DE-STR mass spectrometer.

Pre-mRNA splicing

HeLa cell nuclear extracts were prepared according to previously established protocols and contained ~10 mg/ml total proteins. For preparation of hypomethylated nuclear extracts, HeLa cells in suspension were treated for 48 h with 250 μM MTA followed by a treatment of 24 h with 750 μM MTA. As control, cells were treated with DMSO. AdML (Hernandez and Keller, 1983; Hardy et al., 1984; Simard and Chabot, 2002) and caspase 2 (Côté et al., 2001) splicing substrates were synthesized using T7 and T3 RNA polymerase (Promega) in the presence of CAP analogue and ^{32}P–α-UTP from the corresponding DNA templates linearized with BamHI or XhoI, respectively. Splicing reactions were performed essentially as described previously (Côté et al., 2001). For antibody inhibition of pre-mRNA splicing, nuclear extracts were preincubated with increasing amount of purified antibodies as indicated for 10 min on ice before the splicing reaction. ^{32}P RNA splicing products were separated on polyacrylamide/urea gels and visualized by autoradiography.

Electrophoretic separation of splicing complexes

This procedure was adapted from Konarska and Sharp (1986); 4-μl aliquots were removed from standard splicing reactions at indicated time points and mixed with 1 μl of heparin at 1 mg/ml. 0.5 μl of loading buffer (1× TBE, 20% glycerol, 1% bromophenol blue, and 1% xylene cyanol) was then added, and the samples were loaded on nondenaturing 4% polyacrylamide gels (acrylamide:bisacrylamide = 80:1), which had been preelectrophoresed at 200 V for 30 min in 50 mM Tris-glycine. Electrophoresis was then continued under the same conditions for 4–5 h at RT. The gel was dried and visualized by autoradiography.

We thank Dave Schriemer (University of Calgary, Alberta, Canada) for the mass spectrometry analysis. We are grateful to Greg Matera for helpful and stimulating discussions. We thank Benoit Chabot (Universitè de Sherbrooke, Sherbrooke, Québec, Canada) Edward Chan, Jane Wu (Washington University, St. Louis, MO), and Greg Matera for reagents. We acknowledge the National Cell Culture Center for providing HeLaS9 cells.

This work was supported by grant no. 011291 from The National Cancer Institute of Canada (NCIC) with funds from the Canadian Cancer Society of Canada to S. Richard. J. Côté and F.-M. Boisvert are recipients of a postdoctoral fellowship and a studentship from the NCIC. F. Bachand is the recipient of a studentship from the Canadian Institutes of Health Research (CIHR). C. Autexier holds an FRSQ Chercheur-Boursier and a Boehringer Ingelheim (Canada) Ltd. Young Investigator award with funds from the CIHR and the Cancer Research Society Inc. S. Richard holds an Investigator award from the CIHR and is a recipient of the Terry Fox Young Investigator award from the NCIC.

Submitted: 8 July 2002
Revised: 1 November 2002
Accepted: 1 November 2002

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