Specific combinations of SR proteins associate with single pre-messenger RNAs in vivo and contribute different functions

Petra Björk,[1] ShaoBo Jin,[1] Jian Zhao,[1] Om Prakash Singh,[1] Jan-Olov Persson,[2] Ulf Hellman,[3] and Lars Wieslander[1]

[1]Department of Molecular Biology and Functional Genomics and [2]Department of Mathematics, Stockholm University, SE-106 91 Stockholm, Sweden
[3]Ludwig Institute for Cancer Research, SE-751 24 Uppsala, Sweden

Serine/arginine-rich (SR) proteins are required for messenger RNA (mRNA) processing, export, surveillance, and translation. We show that in *Chironomus tentans*, nascent transcripts associate with multiple types of SR proteins in specific combinations. Alternative splicing factor (ASF)/SF2, SC35, 9G8, and hrp45/SRp55 are all present in Balbiani ring (BR) pre-messenger ribonucleoproteins (mRNPs) preferentially when introns appear in the pre-mRNA and when cotranscriptional splicing takes place. However, hrp45/SRp55 is distributed differently in the pre-mRNPs along the gene compared with ASF/SF2, SC35, and 9G8, suggesting functional differences. All four SR proteins are associated with the BR mRNPs during export to the cytoplasm. Interference with SC35 indicates that SC35 is important for the coordination of splicing, transcription, and 3′ end processing and also for nucleocytoplasmic export. ASF/SF2 is associated with polyribosomes, whereas SC35, 9G8, and hrp45/SRp55 cosediment with monoribosomes. Thus, individual endogenous pre-mRNPs/mRNPs bind multiple types of SR proteins during transcription, and these SR proteins accompany the mRNA and play different roles during the gene expression pathway in vivo.

Introduction

Within the superfamily of proteins containing an arginine/serine-rich (RS) domain, a set of 10 proteins, the serine/arginine-rich (SR) proteins, is conserved in metazoans (for reviews see Graveley, 2000; Sanford et al., 2003). Individual SR proteins (Ring and Lis, 1994; Wang et al., 1996; Jumaa et al., 1999; Longman et al., 2000; Xu et al., 2005; Xiao et al., 2007) or combinations of SR proteins (Longman et al., 2000) are essential in vivo.

The SR proteins are essential splicing factors and have redundant functions in vitro (Fu et al., 1992; Zahler et al., 1992). It is likely that individual SR proteins also have nonredundant functions (Singh and Valcárcel, 2005). SR proteins bind specific RNA sequences (for review see Tacke and Manley, 1999), and certain pre-mRNAs depend on specific SR proteins for splicing (Fu, 1993; Cavaloc et al., 1994). Individual SR proteins can influence splice site selection differently (Wang and Manley, 1995; Zahler and Roth, 1995). SR proteins can also act as splicing repressors (Shin and Manley, 2002; Shin et al., 2004).

In vitro, SR proteins have multiple functions during constitutive splicing. Two different but not mutually exclusive models have been suggested for the function of SR proteins. First, SR proteins bound to exonic splicing enhancers participate in a network of protein–protein interactions through their RS domains (for review see Graveley, 2000). Second, RS domains directly contact splice site signals (Shen and Green, 2004, 2007). The phosphorylated RS domains presumably bind splicing signals that are partially base paired with uridine-rich small nuclear RNAs and promote this base pairing. Multiple RS domain–RNA interactions, including the branch point and the 5′ splice site, are required for splicing.

Correspondence to Lars Wieslander: Lars.Wieslander@molbio.su.se

S. Jin's present address is Dept. of Cell and Molecular Biology, Karolinska Institute, SE-171 77 Stockholm, Sweden.

J. Zhao's present address is Cancer Center Karolinska, Dept. of Oncology and Pathology, Karolinska Institute, SE-171 77 Stockholm, Sweden.

O.P. Singh's present address is Centre for Environmental Studies, North-Eastern Hill University, Shillong 793 022, India.

Abbreviations used in this paper: ANOVA, analysis of variance; ASF, alternative splicing factor; BR, Balbiani ring; Ct-RSF, *C. tentans* repressor splicing factor; CTD, carboxy-terminal domain; hnRNP, heterogeneous nuclear RNP; mRNP, messenger RNP; NPC, nuclear pore complex; TAP, Tip-associated protein.

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SR proteins are important for alternative splicing (for review see Graveley, 2000), and it is possible that alternative splice site choices are decided by which combination of splicing factors, including SR proteins, associate with a pre-mRNA. Differential recruitment of SR proteins to alternatively spliced transcripts supports such a combinatorial model (Mabon and Misteli, 2005).

Spliceosomal assembly is a cotranscriptional process (Wetterberg et al., 2001; Gornemann et al., 2005; Lacadie and Rosbash, 2005; Listerman et al., 2006), and SR proteins are involved in coupling RNA polymerase II transcription to splicing (Das et al., 2007). Transcription and splicing appear to be interdependent. Transcription rate can influence splice site selection (de la Mata et al., 2003; Howe et al., 2003), and SC35 (Lin et al., 2008) and the SR-like protein Npl3 (Dermody et al., 2008) influence transcription elongation.

In addition, SR proteins are important for posttranscriptional processes. Some SR proteins shuttle between the nucleus and the cytoplasm (Cáceres et al., 1998) and facilitate mRNA transport. SRp20 and 9G8 are involved in the export of intronless mRNAs (Huang and Steitz, 2001), and SRp20, 9G8, and alternative splicing factor (ASF)/SF2 are adapters for Tip-associated protein (TAP)/NXF1–dependent mRNA export (Huang et al., 2003; Lai and Tarn, 2004). ASF/SF2 is involved in regulation of mRNA stability (Lemaire et al., 2002) and translation initiation (Michlewski et al., 2008). SR proteins are also involved in the surveillance of mRNA (Zhang and Krainer, 2004; Sato et al., 2008). ASF/SF2 (Li and Manley, 2005) and SC35 (Xiao et al., 2007) are important for maintenance of genome stability. Furthermore, B52/SRp55 in *Drosophila melanogaster* (Rasheva et al., 2006) and SC35 in mice (Xiao et al., 2007) play specific roles in cell cycle control, and SRp38 is involved in neural differentiation in *Xenopus laevis* (Liu and Harland, 2005).

It is not yet established whether individual SR proteins can perform all of the different SR protein functions during splicing and further steps in the biogenesis of individual transcripts or whether several different types of SR proteins must act together during expression of a gene. Because individual SR proteins can restore splicing in S100 extracts, it is conceivable that a single type of SR protein is sufficient for excision of an intron. However, experimental data suggest that multiple independent SR proteins take part in excision of each intron (Shen and Green, 2004; for review see Graveley, 2000). Most experiments that are relevant to this issue have been performed in vitro or used artificial gene constructs. Therefore, it is important to obtain information on which SR proteins associate with endogenous transcripts, when they do so, and when they leave the transcripts.

We have studied the association of individual types of SR proteins with gene-specific pre-messenger RNPs (mRNPs) and mRNPs in vivo. In this study, we have used the experimental advantages of the polytene chromosomes and the Balbiani ring (BR) genes in salivary gland cells of the dipteran *Chironomus tentans*. We show that four types of SR proteins, ASF/SF2, 9G8, SC35, and hrp45/SRp55, bind cotranscriptionally to individual nascent pre-mRNAs in gene-specific combinations. The BR pre-mRNPs bind all four SR proteins but with different distribution in the pre-mRNPs along the active gene. The four SR proteins are associated with the BR mRNPs during intranuclear transport and nucleocytoplasmic export. In the cytoplasm, ASF/SF2 is associated with the mRNA in polyribosomes, whereas 9G8, SC35, and hrp45/SRp55 cosediment with 80-S complexes. Interference with SC35 resulted in a changed distribution of nascent pre-mRNPs on the BR genes and in a decreased export of BR mRNPs through nuclear pore complexes (NPCs). Our data suggest that pre-mRNAs associate with specific combinations of different types of SR proteins cotranscriptionally and that these SR proteins have different functions during subsequent steps of gene expression.

Results

SR proteins associate in specific combinations with nascent pre-mRNPs

Polytene chromosomes from *C. tentans* salivary gland cells were stained with four anti-SR protein antibodies. Antibodies against ASF/SF2, 9G8, SC35, and hrp45/SRp55 were obtained and tested for their specificity (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1).

In the chromosomes, we recorded the staining that was significantly above background. The patterns of well-stained loci were highly reproducible (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1). Each gene locus was significantly stained in all separate experiments, except for minor differences in staining intensity in some loci comparing different animals (Fig. 1, A and B; and Fig. S3). This is most likely the result of minor variations in transcription level comparing different individuals (unpublished data). However, there was no general correlation between staining intensity for the SR proteins and for RNA polymerase II (Fig. 1 C). This is in agreement with the previous finding that labeling intensity for hrp45/SRp55 was not correlated with bromo-UTP incorporation (Singh et al., 2006). Even though staining is evidently influenced by the amount of pre-mRNA, the main factors deciding the staining intensity are gene-specific properties, e.g., pre-mRNA binding specificity for a specific SR protein.

Four main results were obtained. First, each SR protein was present in a large number of gene loci. In total, each SR protein was detected in ∼125 gene loci throughout the polytene chromosomes. In chromosome I (Fig. 1), ∼40 well-stained loci were observed for each antibody.

Second, the staining intensity differed between gene loci in a reproducible way, suggesting that the amount of SR protein associated with pre-mRNA was gene specific (Fig. 1, A and B). Third, there was a high degree of overlap between the staining intensities for all four SR proteins. Therefore, the majority of stained gene loci contained multiple types of SR proteins (Fig. 1, A and B).

Fourth, double-labeling experiments showed that the relative abundance of the different types of SR proteins bound to pre-mRNA was gene specific. In these double-labeling experiments, the relative staining intensities for two SR proteins in the same chromosome were compared. In each gene locus, the two antibodies probed the same number of transcripts.

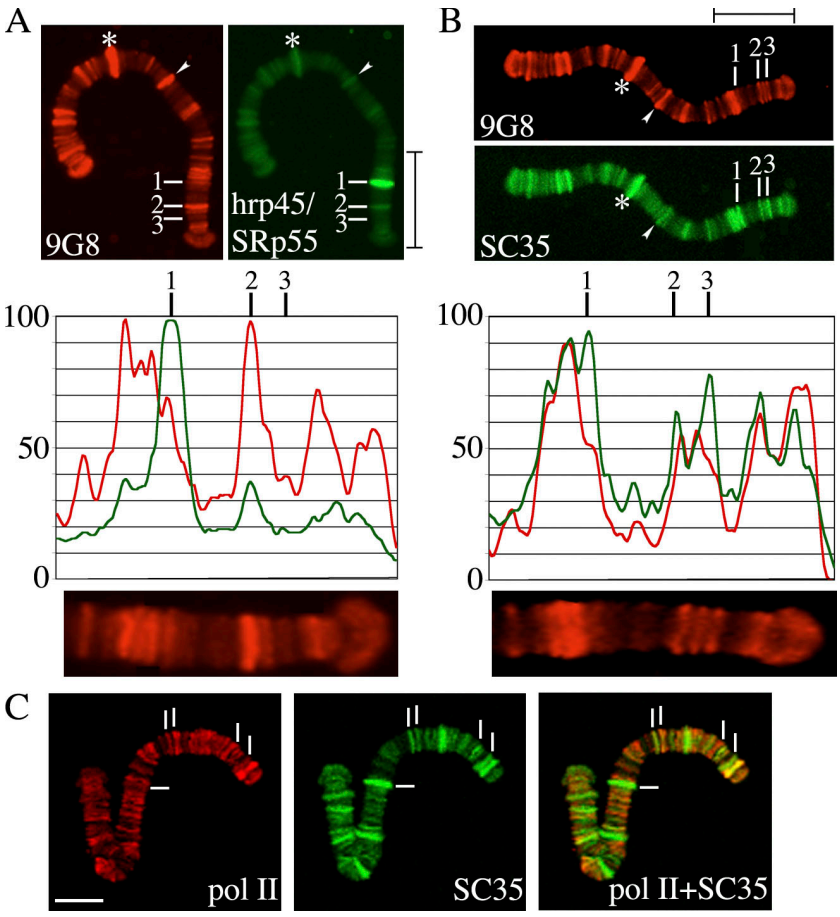

Figure 1. **The relative amount of different types of SR proteins associated with nascent pre-mRNAs is gene specific.** (A and B) Chromosome I immunostained with combinations of anti-9G8 and anti-hrp45/SRp55 antibodies (A) or anti-9G8 and anti-SC35 antibodies (B). The fluorescence intensity of a chromosome I segment (black line) was scanned (A, bottom, 9G8 and hrp45/SRp55; B, bottom, 9G8 and SC35). 9G8 staining is in red, and hrp45/SRp55 and SC35 staining is in green. Enlargements of the scanned segments, which are stained for 9G8, are shown below the diagrams to simplify the interpretation of line scans. 1, 2, and 3 indicate three specific gene loci. The y axis represents the fluorescence intensity in arbitrary units. The arrowheads and asterisks indicate gene loci in chromosome I that contain all four investigated SR proteins (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1). (C) Chromosome I was stained with anti–RNA polymerase II antibody (red) and anti-SC35 antibody (green). The white lines are examples of gene loci that were stained considerably stronger for one or the other protein. Bar, 20 μm.

Assuming that the labeling efficiency for each antibody was the same from locus to locus, the different, even opposite, relative labeling intensities for the two antibodies (comparing two separate loci) indicated that different relative amounts of the two SR proteins were associated with the transcripts in the two gene loci. In Fig. 1 (A and B, bottom), the staining intensities for 9G8 + hrp45/SRp55 and 9G8 + SC35 are shown for a segment of chromosome I. Even though most of the gene loci associated with all three SR proteins, individual loci contained relative differences in the amount of the SR proteins. For example, the loci marked 1 and 2 contained different proportions of 9G8 and hrp45/SRp55 (Fig. 1 A, bottom). By relating the staining intensity for SC35 and hrp45/SRp55 to that for 9G8, it is evident that the gene loci marked 1, 2, and 3 contain different relative amounts of the three SR proteins: hrp45/SRp55 and SC35 > 9G8 in locus 1, 9G8 and SC35 > hrp45/SRp55 in locus 2, and SC35 > 9G8 > hrp45/SRp55 in locus 3 (Fig. 1, A and B, bottom).

ASF/SF2 was present largely in the same gene loci in chromosome I as the other three SR proteins (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1). Also for ASF/SF2, the staining intensity varied in the different gene loci. Because we could not perform double labeling with the ASF/SF2 antibody, it was not possible to accurately compare the amount of ASF/SF2 in relation to the other SR proteins, but the staining intensities indicated that the conclusion for 9G8, SC35, and hrp45/SRp55 is true also for ASF/SF2. As examples, two gene loci (Fig. 1, A and B, arrowheads and asterisks; and Fig. S4 A, arrowheads and asterisk) consistently stained with the opposite intensities compared with 9G8, hrp45/SRp55, and SC35. The amount of ASF/SF2 bound to pre-mRNA in these loci is likely to contribute to a difference in the combination of relative amounts of the SR proteins.

Only a few genes have been characterized in *C. tentans*, and the identification of individual genes is only possible in specific cases in the chromosome preparations. On chromosome IV, the three BR gene loci, BR1, BR2, and BR3 (Wieslander, 1994), contained substantial amounts of all four SR proteins (Fig. 2, A–F; and Fig. S4 A). Quantification of double-stained chromosomes revealed that the BR1 and BR2 genes were very similar and that the BR3 gene contained a relatively higher proportion of SC35. Another gene locus contained 9G8, SC35, and ASF/SF2 but very little hrp45/SRp55 (Fig. 2, A, B, D, and E, arrowheads; and Fig. S4 A, arrowheads).

It is also possible to identify the heat shock genes in chromosome preparations (Sass, 1995). We observed differential binding of SR proteins to heat shock pre-mRNA (Fig. S4 B). In general, we observed that SC35 association with all heat shock pre-mRNAs was consistently much lower than that for 9G8 and hrp45/SRp55.

In conclusion, at least 125 gene-specific nascent pre-mRNPs bound multiple types of SR proteins. In each gene locus, we detected at least three of the four analyzed SR proteins, and in ~90% of the loci, all four SR proteins were present.

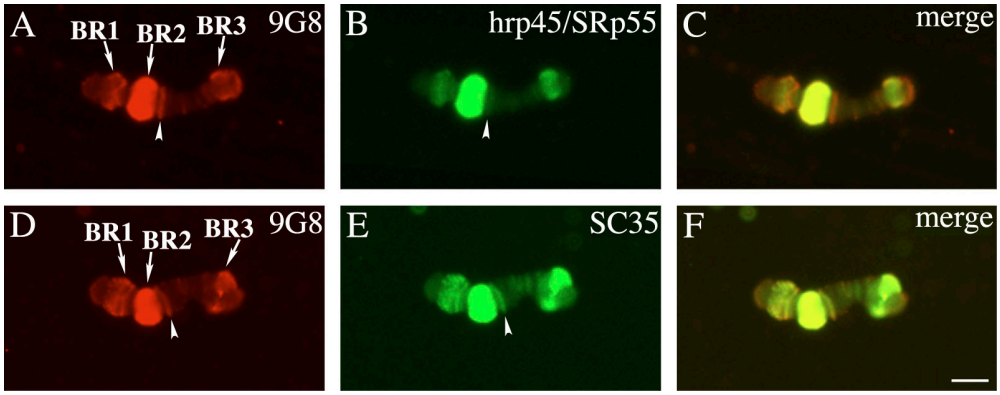

Figure 2. **Different types of SR proteins are associated with BR gene loci.** (A–F) Chromosome IV was immunostained with combinations of anti-9G8 and anti-hrp45/SRp55 antibodies (A–C) or anti-9G8 and anti-SC35 antibodies (D–F). Arrows indicate the active BR1, BR2, and BR3 gene loci. The arrowheads show a fourth gene locus containing 9G8 and SC35 but little hrp45/SRp55. Bar, 10 µm.

The relative abundance of the SR proteins varied in different gene loci. Some loci bound all four SR proteins approximately to the same extent. Others bound little or none of one of the SR proteins. Our results indicate that each nascent transcript binds multiple types of SR proteins and that the relative abundance of these SR proteins is gene specific.

Different types of SR proteins associate with individual nascent BR pre-mRNPs

The immunofluorescence results, including sensitivity to RNase, suggested that each individual transcript binds multiple SR proteins. The association of the SR proteins with BR1 and BR2 pre-mRNPs was further investigated by immuno-EM. The BR1 and BR2 pre-mRNAs associate with various RNA-binding proteins cotranscriptionally and gradually form morphologically characteristic pre-mRNA–protein complexes (Daneholt, 2001), which are referred to as BR pre-mRNPs (see Fig. 4 B). We found that the SR proteins were associated with BR pre-mRNPs at all stages of transcription and packaging. Growing BR pre-mRNPs labeled for each SR protein are shown in Fig. 3 (A–D).

To ascertain whether multiple types of SR proteins are simultaneously present in an individual BR pre-mRNP, we performed double-labeling experiments. The high density of nascent pre-mRNPs often prevented a definite localization of immunogold particles to single transcripts. However, we found clear examples where colocalization to individual pre-mRNPs was evident. In Fig. 3 (E and F), we show BR pre-mRNPs simultaneously labeled with SC35 + 9G8 and hrp45/SRp55 + 9G8. We conclude that an individual BR pre-mRNP associates (at least often) with more than one type of SR protein during transcription and that this was true for both BR1 and BR2 pre-mRNAs.

The SR proteins are differently distributed along the BR genes

We mapped the distribution of SR proteins along the transcribing BR1 and BR2 genes. Based on the morphology of the BR pre-mRNPs as described in Materials and methods and as shown in Fig. 4, it was possible to determine whether a labeled nascent BR pre-mRNP was located on the 5′ proximal, middle, or 3′ distal segment of the gene.

All four SR proteins were present in BR pre-mRNPs located in the three segments of the gene, but apparently to different extents (Table I). The BR1 and BR2 genes have the same gene structure, transcription characteristics, and pre-mRNP morphology. In initial tests, no difference in the distribution of SC35 and 9G8 was found in the BR1 and BR2 genes (unpublished data), as also has been observed for hrp45/SRp55 (Alzhanova-Ericsson et al., 1996). Therefore, we analyzed the BR1 and BR2 genes together. Because the gene segments are of different lengths (Fig. 4 B) and the BR pre-mRNPs are spaced relatively evenly along the BR1 and BR2 genes, we adjusted the values for the length differences to reflect gold labeling/length unit of the gene (Table I). It was clear that the gold labeling/length unit was different in the three segments, with a high labeling/length unit in the proximal segment for all SR proteins. To statistically assess the differences, 99% confidence intervals were calculated using log-transformed data (Table I). The geometric mean values were very similar for SC35, 9G8, and ASF/SF2. However, these values were different for hrp45/SRp55, compared with the other three SR proteins, in the proximal and distal segments.

These results show that the SR proteins are present in the BR pre-mRNPs all along the transcribed gene but that SC35, 9G8, and ASF/SF2 are mainly present in BR pre-mRNPs in the proximal segment and hrp45/SRp55 is especially present in the BR pre-mRNPs in the proximal and distal segments.

Different types of SR proteins are associated with individual BR mRNPs during export

BR mRNPs are morphologically distinct granular structures when they are present in the interchromatin space (Daneholt, 2001). Therefore, we could investigate by immuno-EM whether the SR proteins are bound to the BR mRNPs after release from the genes. We found that BR mRNPs present in the interchromatin space were labeled with antibodies directed against all four SR proteins. Double-labeling experiments showed that two SR proteins were simultaneously labeled in individual BR

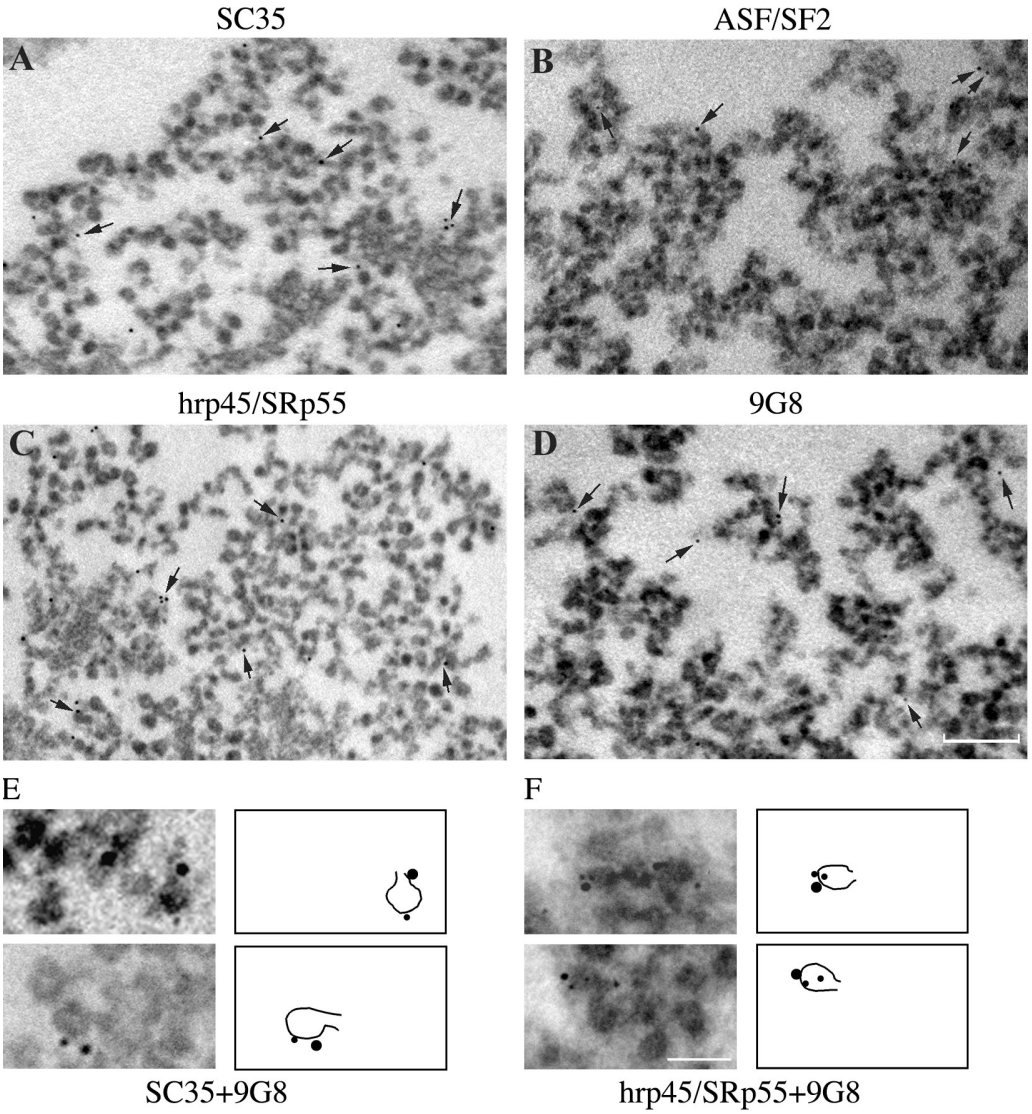

Figure 3. **Different types of SR proteins are associated with BR pre-mRNPs.** (A–D) Chromosome IV was stained with anti-SC35 (A), anti-ASF/SF2 (B), anti-hrp45/SRp55 (C), or anti-9G8 (D) antibodies and analyzed by EM. Gold particles attached to the secondary antibodies show specific labeling of nascent BR pre-mRNPs (arrows). (E and F) Combinations of anti-SC35 (6-nm gold) and anti-9G8 (12-nm gold) antibodies (E) and anti-hrp45/SRp55 (6-nm gold) and anti-9G8 (12-nm gold) antibodies (F) showed that individual BR pre-mRNPs were associated with these combinations of SR proteins. Two separate examples are shown for each combination of antibodies. A schematic representation of the double-labeled individual BR pre-mRNP is shown to the right of each micrograph. Bars: (A–D) 200 nm; (E and F) 100 nm.

mRNPs (Fig. 5, A and B). It seems unlikely that this would happen by chance. The background labeling with unspecific antibodies was ~0.5%, and assuming that the unspecific antibodies bind independently of each other, the probability of unspecific double labeling is 0.0025%. Analyzing 850–900 BR mRNPs for each combination of antibodies, the number of double labeling is binomially distributed, and the probability of unspecifically obtaining the observed number of double-labeled BR mRNPs (20 and 29) was extremely low. Because we do not know how efficiently the antibodies detect their respective epitopes in the EM preparations, our results do not prove that all BR mRNPs contained multiple SR proteins.

We also analyzed BR mRNPs docking at the NPCs. Double labeling of docked BR mRNPs with SC35 + 9G8 (133 BR mRNPs analyzed) and with hrp45/SRp55 + 9G8 (106 BR mRNPs analyzed; Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1) occurred at similar frequencies as double labeling of BR mRNPs in the interchromatin space (see Materials and methods). As for the BR mRNPs in the interchromatin space, the probability of unspecifically obtaining the observed numbers of double-labeled BR mRNPs (5 and 3) was very low. We conclude that multiple types of SR proteins are likely to be associated with individual BR mRNPs during transport through the interchromatin space and when the BR mRNPs interact with the NPCs.

SR proteins are associated with mRNA in the cytoplasm

Immuno-EM demonstrated that each one of the four SR proteins was part of the BR mRNPs on the cytoplasmic side of the

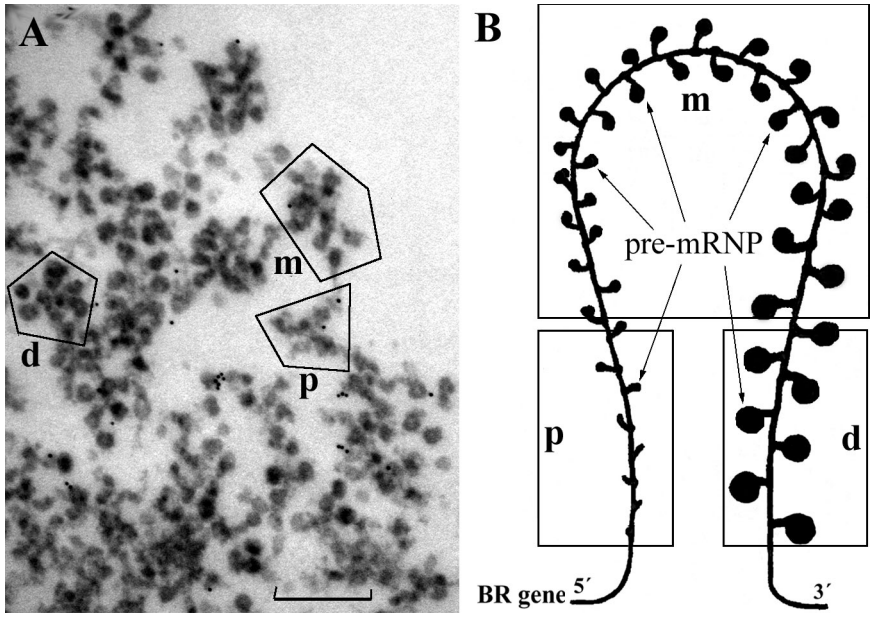

Figure 4. **The SR proteins are differently distributed along active BR genes.** Chromosome IV was stained with anti-SC35, anti-9G8, anti-ASF/SF2, or anti-hrp45/SRp55 antibodies and analyzed by EM. For each SR protein, all gold particles were attributed to proximal (p), middle (m), or distal (d) segments of the BR gene (Table I). (A) A section through a BR gene locus stained with anti-hrp45/SRp55 antibody is shown to illustrate proximal, middle, and distal gene segments. (B) An active BR gene with growing BR pre-mRNPs is shown schematically based on EM reconstruction data (Daneholt et al., 1982). The three segments (p, m, and d) of the active gene are indicated. Each segment is characterized by the morphology of the BR pre-mRNPs. Bar, 200 nm.

NPCs during translocation (Fig. 5, C–F), indicating that they accompanied the BR mRNPs into the cytoplasm. Immuno-EM showed significant labeling for the SR proteins in the cytoplasm of salivary gland cells (unpublished data), but the BR mRNPs are not morphologically identifiable in the cytoplasm. Therefore, we used *C. tentans* epithelial tissue culture cells to show that ASF/SF2, SC35, 9G8, and hrp45/SRp55 were all found in the cytoplasm (Fig. 6, A and B, input lanes) and used UV cross-linking experiments to show that the cytoplasmic SR proteins were bound to cytoplasmic mRNA (Fig. 6 A). SC35 in *C. tentans* and *D. melanogaster* lacks the nuclear retention sequence present at the C terminus of mammalian SC35 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1). This is in agreement with the fact that we found SC35 in the cytoplasm.

To analyze whether the SR proteins are associated with the mRNA during translation, we fractionated cytoplasmic extracts from epithelial tissue culture cells on sucrose gradients and analyzed the different fractions for the presence of individual types of SR proteins by Western blotting. Only ASF/SF2 was

Table I. **Distribution of immunogold labeling of SR proteins in the proximal, middle, and distal segments of the BR genes**

SR protein/experiment	Proximal	Middle	Distal
SC35			
1	0.52	0.29	0.19
2	0.48	0.35	0.17
3	0.52	0.31	0.17
mean (c.i.)	0.51 (0.39–0.66)	0.11 (0.06–0.18)	0.17 (0.13–0.24)
9G8			
1	0.53	0.28	0.19
2	0.51	0.29	0.20
3	0.59	0.27	0.14
mean (c.i.)	0.54 (0.35–0.83)	0.09 (0.07–0.12)	0.17 (0.06–0.48)
ASF/SF2			
1	0.58	0.29	0.13
2	0.62	0.30	0.08
3	0.52	0.32	0.16
mean (c.i.)	0.57 (0.35–0.94)	0.10 (0.08–0.13)	0.12 (0.02–0.81)
hrp45/SRp55			
1	0.30	0.31	0.39
2	0.32	0.30	0.38
3	0.29	0.33	0.38
mean (c.i.)	0.30 (0.25–0.38)	0.10 (0.09–0.13)	0.38 (0.38–0.39)

c.i., confidence interval. Three independent experiments are listed for each SR protein. For each experiment, the proportions of gold labeling in the three gene segments are shown. In each experiment, 300–750 gold particles were analyzed for SC35, 9G8, and hrp45/SRp55, and 100–200 gold particles were analyzed for ASF/SF2. The geometric mean and 99% confidence interval for relative distributions of gold labeling/length unit are shown. Data were adjusted for length differences between the proximal, middle, and distal gene segments and were log transformed.

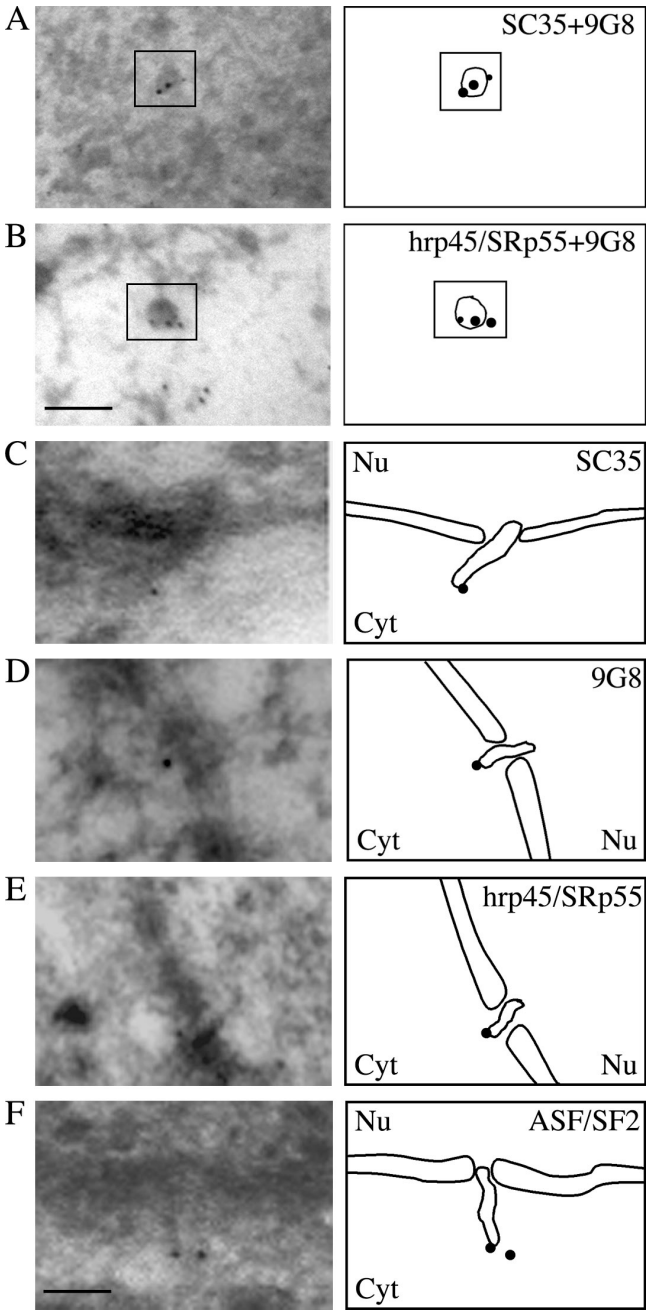

Figure 5. **Different types of SR proteins are associated with BR mRNPs during nucleocytoplasmic transport.** (A and B) Sections through salivary gland nuclei were stained with combinations of anti-SC35 and anti-9G8 antibodies (A) or with anti-hrp45/SRp55 and anti-9G8 antibodies (B). Examples of BR mRNPs associated with SC35 (12-nm gold) and 9G8 (6-nm gold) or with hrp45/SRp55 (12-nm gold) and 9G8 (6-nm gold) in the interchromatin space are shown. (C–F) Examples are shown of BR mRNPs associated with SC35 (C), 9G8 (D), hrp45/SRp55 (E), or ASF/SF2 (F) during translocation through NPCs. In each case, the gold particle was associated with the part of the BR mRNP that had reached the cytoplasm (Cyt). Schematic interpretations are shown to the right of each micrograph. Nu, nucleus. Bars, 100 nm.

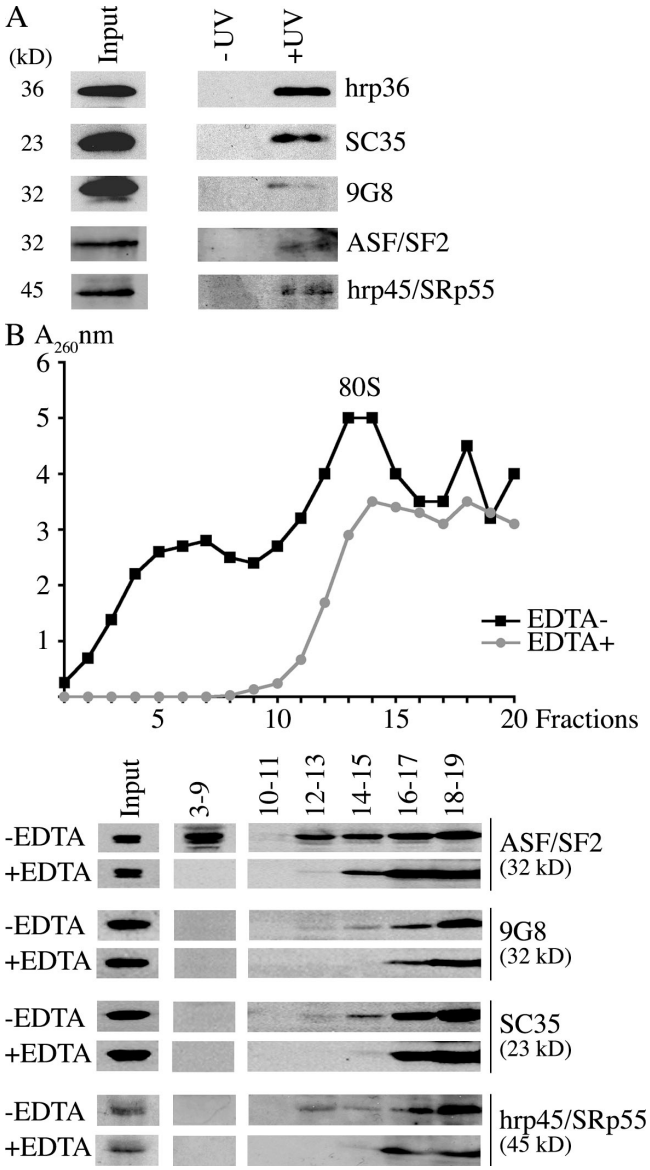

Figure 6. **Different types of SR proteins are associated with mRNA in the cytoplasm, but only ASF/SF2 is associated with the mRNA in polyribosomes.** (A) SC35, 9G8, ASF/SF2, and hrp45/SRp55 copurified with cytoplasmic poly(A)$^+$ RNA after UV cross-linking (+UV) but not in the absence of UV cross-linking (−UV). The hnRNP A/B-type protein hrp36 served as a positive control. (B) ASF/SF2 was associated with polyribosomes, whereas 9G8, SC35, and hrp45/SRp55 cosedimented with monoribosomes. Cytoplasmic extracts treated (+) or not treated (−) with EDTA were fractionated in 15–50% sucrose gradients. The absorbance at 260 nm showed EDTA sensitivity for both polyribosomes and 80-S monoribosomes. Fractions from the sucrose gradients were pooled as indicated and subjected to Western blot analyses for detection of the indicated proteins. (A and B) The relative molecular mass is indicated for each SR protein.

associated with polyribosomes in an EDTA-sensitive manner (Fig. 6 B). SC35, 9G8, and hrp45/SRp55 were detected in the 60–80-S part of the gradient but not in the polyribosome part (Fig. 6 B). EDTA treatment of the extracts shifted SC35, 9G8, and hrp45/SRp55 from the 60–80-S to the 40–60-S part of the gradient. The EDTA concentration used is likely to dissociate ribosomes into subunits but not affect mRNPs (Calzone et al., 1982).

Our data show that ASF/SF2, SC35, 9G8, and hrp45/SRp55 were bound to mRNA in the cytoplasm and that ASF/SF2 was associated with mRNA during translation. SC35, 9G8, and hrp45/SRp55 were not present in polyribosomes, but our data are consistent with the possibility that they were associated with mRNPs that were bound to a single ribosome.

SC35 influences the distribution of nascent BR pre-mRNPs on the BR genes

To analyze the importance of SC35 for BR mRNP biogenesis, we interfered with SC35 function in vivo. We injected anti-SC35 antibody into the cell nuclei of salivary gland cells and analyzed the effect on synthesis of BR mRNPs. Compared with controls (cells injected with unrelated antibody or uninjected cells), the number and length of gene loops were apparently not altered, and the genes had pre-mRNPs on the proximal, middle, and distal segments. Thus, we did not observe any significant change in the overall characteristics of the transcribing BR gene loops when interfering with SC35 function in vivo.

The morphology of the individual BR pre-mRNPs was not significantly affected. Therefore, the packaging of the nascent transcript with heterogeneous nuclear RNPs (hnRNPs) and other proteins was not influenced at the level of resolution investigated.

We analyzed the distribution of pre-mRNPs on the proximal, middle, and distal segments of transcribing BR1 and BR2 genes (Table II). The gene segments were identified as described in Materials and methods. In both types of control cells, we found the expected distribution of pre-mRNPs along the BR genes (i.e., ∼20% in the proximal, 60% in the middle, and 20% in the distal segments). Thus, the BR pre-mRNPs were approximately evenly distributed along the entire gene, which is in agreement with a previous study (Lamb and Daneholt, 1979). In cells injected with anti-SC35 antibody, the distribution of BR pre-mRNPs was altered (Table II). To assess whether the proportions of pre-mRNPs in the gene segments were different in controls and anti-SC35 antibody–injected cells, a one-way analysis of variance (ANOVA) was performed on each segment separately, assuming a normal distribution and the same variances (Table II). In particular for the middle and distal segments, there were differences between controls and anti-SC35 antibody–injected cells. With a multivariate ANOVA, in which we simultaneously analyzed the entire distribution, the differences were also significant ($P = 0.0023$).

These results show a relative increase of almost complete transcripts in the distal segment of the gene, a relative decrease in the middle segment, and possibly a slight increase in the proximal segment when interfering with SC35 function in vivo. In the EM sections, it was not possible to directly measure the spacing of the pre-mRNPs, but a modest changed spacing of the BR pre-mRNPs was compatible with the observed morphology. These data suggest that SC35 is involved in processing events that influence transcription elongation rate and/or the release of the mRNP from the gene.

Table II. **Distribution of BR pre-mRNPs in the three BR gene segments after injection of control antibody or anti-SC35 antibody**

Injection/ experiment	Proximal	Middle	Distal
Uninjected			
1	0.22	0.56	0.22
2	0.24	0.53	0.23
3	0.23	0.54	0.23
mean (c.v.)	0.23 (0.061)	0.54 (0.037)	0.23 (0.028)
Control antibody			
1	0.25	0.51	0.24
2	0.24	0.54	0.22
3	0.24	0.55	0.21
mean (c.v.)	0.24 (0.025)	0.53 (0.046)	0.22 (0.087)
SC35 antibody			
1	0.25	0.41	0.34
2	0.27	0.40	0.33
3	0.25	0.39	0.36
mean (c.v.)	0.26 (0.060)	0.40 (0.027)	0.34 (0.042)

c.v., coefficient of variation. Three independent experiments for each treatment are listed: uninjected cells, cells injected with control antibody, and cells injected with anti-SC35 antibody. For each experiment, the relative distribution of BR pre-mRNPs and one-way ANOVA analyses of log-transformed data are shown. Geometric mean and coefficient of variation are listed. P-values from one-way ANOVA are as follows: proximal, $P = 0.095$; middle, $P = 0.00008$; and distal, $P = 0.0001$.

SC35 is important for BR mRNP transport in vivo

We analyzed the export of the BR mRNPs in cells injected with anti-SC35 antibody compared with injection with anti–*C. tentans* repressor splicing factor (Ct-RSF) antibodies (Fig. 7 and Table III). Ct-RSF, an RNA-binding protein that can repress splicing (Björk et al., 2006), is present in the BR mRNPs during transport (Sun et al., 1998). In the preparations used, we could accurately identify the BR mRNPs docked at the basket of the NPC. Based on previous characterizations (Soop et al., 2005), we defined the BR mRNPs as docked when they were present within 50 nm from the nuclear membrane. At these docking sites, we could observe NPC structures. The injection of anti-SC35 antibody resulted in a significant increase in the number of docked BR mRNPs at NPCs (Table III). Consistent with this finding, the number of BR mRNPs in the interchromatin space increased in the injected cells compared with the control cells. We conclude that interfering with SC35 in vivo resulted in impaired export of the BR mRNPs and in an accumulation of BR mRNPs in the nucleus.

Discussion

We report data on the association of four different types of SR proteins with a large set of pre-mRNPs transcribed from endogenous genes. We further describe the association of these SR proteins with pre-mRNPs and mRNPs derived from the BR genes, whose exon–intron structure and splicing characteristics are known. Therefore, we can give a detailed picture of the association of the SR proteins with specific pre-mRNPs and mRNPs in vivo during transcription, splicing, and export to the cytoplasm.

Pre-mRNPs associate with several different types of SR proteins cotranscriptionally in gene-specific combinations

Our data suggest that although most gene loci contained all four analyzed SR proteins, there was a gene-specific combination of the abundance of the proteins. How can we explain this finding, and what is the functional implication? Because the gene-specific combinations were observed in the same cell, it

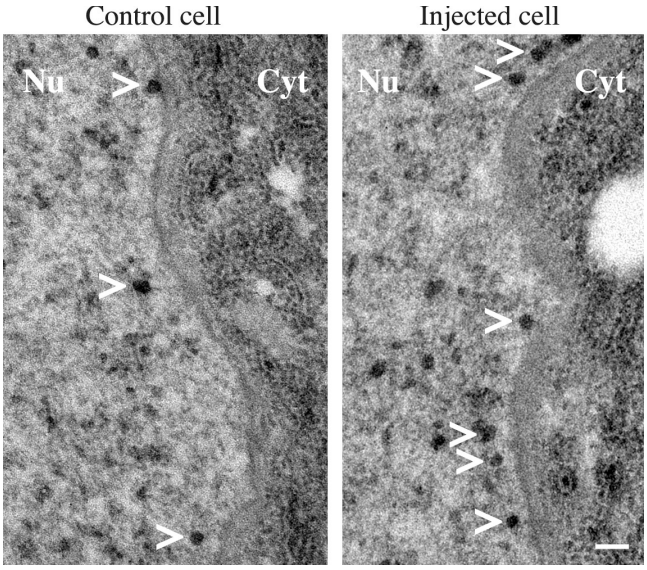

Figure 7. **SC35 is involved in export of BR mRNPs.** The export of BR mRNPs was analyzed in control cells injected with anti–Ct-RSF antibodies (left) and in cells injected with the anti-SC35 antibody (right). BR mRNPs docked at NPCs (arrowheads) were recorded. Nu, nucleus; Cyt, cytoplasm. Bar, 100 nm.

is likely that gene-specific properties determined the SR protein association. SR proteins have substrate specificity defined by their RNA recognition motif domain (Mayeda et al., 1999). RNA sequence motifs with some SR protein binding specificity have also been identified (Sanford et al., 2008; for review see Tacke and Manley, 1999). It is likely that natural pre-mRNAs contain combinations of such degenerate sequence motifs (Liu et al., 1998). We assume that these binding sequences on the pre-mRNAs determine the gene-specific SR protein association patterns. This is in accordance with a previous finding that SRp20 associates with a subset of transcription sites in mammalian cells (Neugebauer and Roth, 1997). In addition, other proteins presumably influence the binding of SR proteins. For example, binding of hnRNP proteins occurs cotranscriptionally and independent of splicing factors and could influence SR protein binding (Amero et al., 1992). Furthermore, an hnRNP M protein, hrp59, binds specific pre-mRNAs at exon sites that may overlap with SR protein binding and may therefore interfere with splicing regulation (Kiesler et al., 2005). In functional terms, it is possible that the specific combination of SR proteins that is bound to an mRNA influences the efficiency of expression because the SR proteins act at several different steps such as processing, transport, and translation. It is even possible that the expression of sets of mRNAs is optimized in a coordinated manner. We found that hrp45/SRp55 binds pre-mRNA at many gene loci, although, as previously reported using a different labeling method, the loci with the highest concentration of hrp45/SRp55 included several genes expressing salivary gland secretory proteins (Singh et al., 2006). These genes are not only highly expressed but also belong to the same gene family (Wieslander, 1994). Because the intensity of labeling is not correlated with the amount of nascent RNA (Singh et al., 2006), it is likely that hrp45/SRp55-binding sites are more abundant in efficiently expressed gene transcripts. It has been suggested that ASF/SF2 binds to and coregulates processing and translation of specific mRNAs (Sanford et al., 2008).

SR proteins are highly mobile within the nucleus (Phair and Misteli, 2000) and are recruited to nascent pre-mRNPs as shown here and elsewhere (Misteli et al., 1998; Björk et al., 2006). Recruitment requires hyperphosphorylation, and, possibly, SR proteins are recruited to the pre-mRNP via the transcribing RNA polymerase II (Das et al., 2007), but the mechanism for recruitment in vivo is not known. It has been proposed that formation of spliceosomes is stochastic and that spliceosomes contain different combinations of splicing factors (Mabon and Misteli, 2005). Our data show that the combination of SR proteins on a given pre-mRNP is reproducible. This argues that binding sites on the pre-mRNPs influence the dwell time for the bound SR proteins and thus the combination of SR proteins associated with a given pre-mRNP at a given time. In the BR genes, our data do not rule out the possibility that individual spliceosomes may involve different combinations of the four SR proteins.

Several different types of SR proteins are present in BR pre-mRNPs when individual introns are excised

Expression of the BR genes has been analyzed in detail (Baurén and Wieslander, 1994; Baurén et al., 1998; Daneholt, 2001;

Table III. **Effect of injection of anti-SC35 antibody on BR mRNPs in interchromatin space and docked at NPCs**

Injection/experiment	Interchromatin space: injected/uninjected	NPC: injected/uninjected
Control antibody		
1	1.11	1.16
2	0.84	0.91
mean (c.v.)	0.97 (0.20)	1.03 (0.19)
SC35 antibody		
1	1.50	1.75
2	1.63	2.00
3	2.24	2.00
mean (c.v.)	1.77 (0.21)	1.91 (0.08)

c.v., coefficient of variation. Ratios between injected and uninjected cells in the same gland are listed for two independent control experiments (injected with anti–Ct-RSF antibodies) and three independent injections of anti-SC35 antibody. The geometric mean and coefficient of variation of the ratios are shown. P-values from *t* test on log-transformed data are as follows: interchromatin space, P = 0.05; and NPC, P = 0.01.

Wetterberg et al., 2001). Therefore, we can uniquely relate the association of the different SR proteins to BR gene structure, transcription, and processing in vivo. The BR1 and BR2 genes have three introns within the first 3 kb of the gene and a fourth intron located downstream of the 35-kb-long exon 4 and ∼600 bp from the polyadenylation site (Wieslander, 1994). The BR3 gene contains 38 short introns evenly distributed in the 11-kb gene. The BR1, BR2, and BR3 genes encode secretory proteins. They are efficiently expressed, and their pre-mRNAs are constitutively spliced. All four SR proteins associated extensively with BR1, BR2, and BR3 pre-mRNAs, regardless of the large difference in intron numbers and thus the number of splicing reactions. It is evident that a single type of SR protein is involved in excision of many introns in the BR3 pre-mRNAs.

Several SR protein molecules are needed during constitutive splicing. This conclusion is based on the multitude of demonstrated protein–protein interactions that SR proteins are involved in (for review see Graveley, 2000) and on the fact that more than one SR protein molecule contacts the pre-mRNA during splicing (Shen and Green, 2004, 2007). In vitro, a single type of SR protein is sufficient for splicing. Therefore, it is conceivable that this is possible also in vivo.

The four investigated SR proteins, including hrp45/SRp55 (Björk et al., 2006), can support splicing in vitro. When analyzing the distribution of the SR proteins along the BR genes, we consider that the most interesting aspect is the presence of each SR protein/pre-mRNP, which is approximately equivalent to SR protein/length unit of the gene. From these estimates, we conclude that ASF/SF2, SC35, and 9G8 were enriched in pre-mRNPs in the proximal 7-kb segment of the BR1 and BR2 genes. This correlates as to location and time with the cotranscriptional splicing of the three proximal introns (Baurén and Wieslander, 1994). Although the SR proteins were also present in the intronless middle segment as previously reported for hrp45/SRp55 (Alzhanova-Ericsson et al., 1996), the amount of the SR proteins, especially for SC35, ASF/SF2, and 9G8, declined in the middle segment of the gene. We cannot exclude the possibility that conformational changes influenced recognition of epitopes differently along the gene. If so, this would affect the SR proteins somewhat differently, and, furthermore, the accessibility would tend to increase again in the distal segment. We favor the interpretation that particularly the ASF/SF2, SC35, and 9G8 proteins leave the BR pre-mRNP to a considerable extent after completion of excision of introns 1–3 and that additional recruitment of the proteins occurs at the distal segment. This would be compatible with previous findings that substantial amounts of molecular mass are added to and lost from the transcription-splicing complex during cotranscriptional splicing (Wetterberg et al., 2001). In the present study, our results suggest that SR protein association with pre-mRNP is dynamic in relation to the splicing process.

Intron 4 in the BR1 and BR2 genes is present in the distal segment and is cotranscriptionally removed in ∼10% of the transcripts. Our results indicate that hrp45/SRp55 is relatively more enriched in the distal segment compared with the other three SR proteins. Therefore, all four SR proteins, and in particular hrp45/SRp55, are present when intron 4 excision is initiated. In summary, although four types of SR proteins are present in the BR pre-mRNPs when introns 1–3 and intron 4 are excised, our data suggest that the proportions of the SR proteins differ during these splicing events.

Individual types of SR proteins have multiple functions during expression of individual genes

Our functional experiments of SC35 show that individual types of SR proteins have several different functions in the cell nucleus during processing and export of a single mRNP. On the BR genes, interference with SC35 function did not block initiation and elongation of transcription nor release of BR mRNPs from the genes, but the distribution of nascent BR pre-mRNPs changed. The simultaneous decrease of transcripts in the ∼20-kb-long middle segment, increase in the distal segment, and no change or a tendency for an increase in the proximal segment are compatible with a relatively faster elongation rate within the middle segment. The proximal segment contains three introns. There is one intron in the distal segment but no introns in the middle segment. The three 5′-located introns are to a large extent removed before the RNA polymerase II has reached the middle segment. Intron 4 is excised cotranscriptionally in ∼10% of the transcripts (Baurén and Wieslander, 1994). Interestingly, it has recently been shown that SC35 in mammalian cells recruits the kinase positive transcription elongation factor b to the elongation complex. This kinase is important for RNA polymerase II carboxy-terminal domain (CTD) serine 2 phosphorylation and thus for elongation (Lin et al., 2008). Also, the yeast SR-like protein Npl3 interacts with serine 2 of the CTD and stimulates elongation of RNA polymerase II (Dermody et al., 2008). Therefore, it is possible that our data on interference with SC35 function reflect a negative effect on transcription elongation in the proximal and distal segments where splicing machineries are assembled and operate.

Our data showed the most drastic effect on nascent transcripts in the distal segment of the BR gene. Because positive transcription elongation factor b phosphorylation of CTD serine 2 is required for efficient 3′ end processing (Ahn et al., 2004; Ni et al., 2004), inhibition of SC35 could indirectly influence 3′ end processing. In addition to a coordination between SC35 and transcription and 3′ end processing via CTD phosphorylation at serine 2 residues, there is a coupling between excision of the last intron and 3′ end cleavage and polyadenylation (Vagner et al., 2000; Kyburz et al., 2006; Rigo and Martinson, 2008). In the BR1 gene, transcription extends 600 bp downstream of the polyadenylation site. At this position, transcription, excision of intron 4 and 3′ end cleavage, and polyadenylation occur at the same time (Baurén et al., 1998). Therefore, impairment of SC35 function during excision of intron 4 could affect the release of the pre-mRNPs from the gene. An alternative mechanism could be that SC35 has a function analogous to Npl3p. This yeast protein is associated with the nascent transcript and must be dephosphorylated to release mRNP from the 3′ end processing machinery and allow association with the mRNA export receptor Mex67p (Gilbert and Guthrie, 2004).

SC35 was associated with BR mRNPs in the interchromatin space, in which splicing of the transcripts is essentially complete (Baurén and Wieslander, 1994). BR mRNPs as well as other mRNPs (Politz et al., 1999; Singh et al., 1999; Shav-Tal et al., 2004) move by diffusion through the interchromatin space and subsequently dock at the basket of the NPCs. We showed that interfering with SC35 partially inhibited export through the NPC, leading to an increase of BR mRNPs in the nucleus. BR mRNPs are delivered from the genes to the interchromatin space, and a pool of BR mRNPs is present in the interchromatin space. This pool is approximately constant in size and completely turned over during 90–120 min (Singh et al., 1999). Based on these facts, we estimate that the block in export is partial and in the range of 20–25%. Our data indicate that the negative effect on export through the NPCs is more prominent than a possible negative effect on release of BR mRNPs into the interchromatin space. Based on previous analyses of BR mRNP export through NPCs (Soop et al., 2005), our data show that the accumulation of BR mRNPs after SC35 interference occurs at the stage of initial binding at the NPC.

Our data are compatible with a role for *C. tentans* SC35 as an export adapter, perhaps for the general mRNA export factor TAP/NXF1. It has previously been shown that dephosphorylated forms of ASF/SF2, 9G8, and SRp20 act as export adapters for TAP/NXF1 (Huang et al., 2003; Lai and Tarn, 2004). Because ASF/SF2, 9G8, and hrp45/SRp55 are also part of the BR mRNPs in the interchromatin space and at the NPCs, it is possible that several SR proteins contribute to the export of an individual BR mRNP.

Different association of SR proteins with mRNPs during translation

Our data show that the four types of SR proteins are associated with mRNAs in the cytoplasm, suggesting that these SR proteins have additional functions there. Only ASF/SF2 was found in polyribosomes, which is consistent with a role during translation. In agreement with these data, ASF/SF2 is also known to be associated with polyribosomes in mammalian cells, where it has been shown to stimulate cap-dependent translation (Michlewski et al., 2008).

Additional studies have also shown that SR proteins have functions connected to translation. SRp20 functions in internal ribosome entry site–mediated translation of viral RNAs (Bedard et al., 2007). In yeast, the SR-like proteins Npl3p, Gbp2p, and Hrb1p are involved in translation initiation (Windgassen et al., 2004). 9G8 stimulates the association of unspliced RNA that contains constitutive transport elements with polyribosomes (Swartz et al., 2007), and the protein was also found in light polyribosomes.

We detected 9G8, SC35, and hrp45/SRp55 only in the 80-S ribosome fractions. It appears that these SR proteins dissociated from the mRNPs before cap-dependent translation in polyribosomes. Therefore, it is possible that these three SR proteins are involved in translation initiation or possibly in a first round of translation connected to non-sense–mediated decay (Zhang and Krainer, 2004). In either case, the different behavior compared with ASF/SF2 indicates differences in function for the different SR proteins.

Materials and methods

Biological material

Animals and cells. *C. tentans* was cultured as previously described (Meyer et al., 1983). A *C. tentans* embryonic epithelial cell line was cultured as previously described (Wyss, 1982). For heat shock treatment, larvae were kept at 37°C for 60 min.

Antibodies. SR proteins were purified from *C. tentans* (Fig. S1 B) and injected into mice. A monoclonal cell line producing anti-SC35 antibody was obtained by standard procedures (Mabtech).

C. tentans 9G8 protein was produced using the BacPAK Baculovirus Expression System (Clontech Laboratories, Inc.). Polyclonal antibodies were raised in rabbits (AgriSera AB). The 9G8-specific antibodies were purified by chromatography on cyanogen bromide–activated Sepharose 4B columns (GE Healthcare), to which 9G8 had been coupled.

The mAb104 antibody (Roth et al., 1990; Zahler et al., 1992) was a gift from M.B. Roth (Fred Hutchinson Cancer Research Center, Seattle, WA). Anti-ASF/SF2 monoclonal antibody was a gift from A.R. Krainer (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) or was obtained from Invitrogen. Monoclonal antibody against hrp45 and hrp36 was a gift from B. Daneholt (Karolinska Institute, Stockholm, Sweden). hrp45 is the *C. tentans* homologue to mammalian SRp55. The specificities of the anti–SR protein antibodies were checked by Western blotting (Fig. S1 A). The monoclonal anti–RNA polymerase II antibody was purchased from Abcam, and the monoclonal anti–human von Willebrand factor antibody was purchased from Dako. Monoclonal mouse IgG was obtained from Mabtech. The secondary antibodies used for Western blots were swine anti–rabbit Ig HRP and goat anti–mouse Ig HRP (Dako), both diluted 1:3,000. The secondary antibodies used in immunofluorescence were rabbit anti–mouse Ig FITC, swine anti–rabbit Ig FITC (Dako), donkey anti–mouse IgG Texas red (Jackson ImmunoResearch Laboratories), goat anti–rabbit IgG Cy5, and donkey anti–mouse IgG Cy5 (GE Healthcare), all diluted 1:100. The secondary antibodies used in immuno-EM were goat anti–mouse IgG labeled with colloidal gold (diameter of 6 nm or 12 nm) and goat anti–rabbit IgG labeled with colloidal gold (diameter of 6 nm or 12 nm; Jackson ImmunoResearch Laboratories).

Cloning procedures

C. tentans ASF/SF2 and SC35 cDNAs were isolated by RT-PCR using degenerate oligonucleotide primers corresponding to sequences in the homologous genes in *D. melanogaster*. Poly(A)$^+$ RNA was isolated from *C. tentans* epithelial tissue culture cells and reverse transcribed using oligo dT priming. The degenerate oligonucleotide primers were used for PCR, and the obtained PCR fragments were sequenced. Complete cDNA sequences were isolated from a *C. tentans* λ Zap cDNA library.

C. tentans 9G8 was found in a two-hybrid screen using Ct-RSF (Björk et al., 2006) as bait (Matchmaker Two-Hybrid System; Clontech Laboratories, Inc.). All sequencing reactions were performed with the DYEnamic ET Terminator Cycling Sequencing premix kit (GE Healthcare) and analyzed on an Automated DNA Sequencer (model 373A; Applied Biosystems). The sequences of DNA and proteins were analyzed by programs in the Genetics Computer Group package (Devereux et al., 1984) and the Biology Workbench package (http://workbench.sdsc.edu).

Protein preparation, Western blotting, and sequence analysis

SR proteins from *C. tentans* epithelial tissue culture cells (Fig. S1 B) were prepared as described previously (Zahler et al., 1992). Proteins were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride filters by semidry electrophoresis. HRP-labeled secondary antibodies were detected by the ECL method (GE Healthcare).

For amino acid sequence analyses, the protein of interest was treated for in-gel digestion (Hellman, 2000). In brief, the sample was washed with protease-specific buffer (ammonium bicarbonate for trypsin and Tris-HCl for LysC) containing acetonitrile and was thereafter dried under a stream of nitrogen. A solution of trypsin (porcine, modified, sequence grade from Promega) or LysC (prepared from *Achromobacter lyticus*; Wako Chemicals USA, Inc.) was adsorbed into the gel pieces overnight. The reaction was stopped by acidification with trifluoroacetic acid. Generated peptides were extracted and isolated by microbore reversed phase liquid chromatography using a SMART System (GE Healthcare) on a 1 × 150–mm column (Kromasil C18; Column Engineering). Individual peptides were analyzed by Edman degradation on a 494–amino acid sequencer (Applied Biosystems) according to the manufacturer's instruction.

Immunofluorescence analyses and immuno-EM on isolated chromosomes
Polytene chromosomes were isolated and processed for immunofluorescence or EM as described previously (Björk and Wieslander, 2008). For double labeling of RNA polymerase II and SC35, the antibodies were labeled using Zenon Mouse IgG labeling (Invitrogen). Significant staining above background, defined as staining with an unrelated antibody or with only secondary antibody, was recorded. Preparations for immunofluorescence analyses were mounted in Vectashield mounting medium (Vector Laboratories) and viewed in a confocal microscope system (LSM 510; Carl Zeiss, Inc.) equipped with a microscope (Axiovert 100M; Carl Zeiss, Inc.). Images were collected using a 40× oil immersion 1.0 NA Plan-Apochromat lens (Carl Zeiss, Inc.) at room temperature and LSM 510 software (Carl Zeiss, Inc.). Line scans were obtained using ImageJ software (National Institutes of Health). EM specimens were photographed in a 120-kV electron microscope (at 80 kV; Tecnai; FEI) using a charge-coupled device camera (1000P; Gatan) and the DigitalMicrograph acquisition software (Gatan). Images were processed using Photoshop version 8.0 (Adobe).

In sections through active BR gene loci, segments of the BR genes were classified as proximal, middle, or distal based on the structure of the pre-mRNPs (Fig. 4; Daneholt et al., 1982; Daneholt, 2001). In the proximal segment, the pre-mRNPs form fibers lacking distinct granules at their end. In the middle segment, the pre-mRNPs have stalks with distinct granules. These granules get bigger along the segment reaching a diameter of 350 nm. In the distal segment, the pre-mRNPs are dominated by a more compact granule with a diameter of 350–500 nm. These morphological criteria are reproducible, and in reconstructions of complete BR gene loops, the proximal, middle, and distal segments constitute ∼20%, 60%, and 20%, respectively. In sections, immunogold particles, after staining with the anti–SR protein antibodies, were assigned to the three gene segments (identified by applying the aforementioned morphological criteria) for each antibody. The distribution of the immunogold particles between the three segments was calculated. Three independent experiments were performed for each antibody. The total number of counted gold particles was 1,244 for SC35, 978 for 9G8, 210 for ASF/SF2, and 761 for hrp45/SRp55.

To measure the specificity of immunolabeling, we counted the number of immunogold particles in a total area of 25 μm^2 within active BR gene loci for each antibody. We recorded ∼10 gold particles/μm^2 for SC35, hrp45/SRp55, and 9G8 and 5 gold particles/μm^2 for ASF/SF2. The number of immunogold particles in a total area of 25 μm^2 after staining with only secondary antibody or with the unrelated monoclonal anti–von Willebrand antibody plus secondary antibody was ∼0.4 gold particles/μm^2. Thus, the signal/background ratios were 25 for SC35, hrp45/SRp55, and 9G8 and 10 for ASF/SF2.

Immuno-EM on cryosections
Preparations of ultra thin cryosections were performed as described previously (Visa et al., 1996). The grids were immunostained as described previously (Björk and Wieslander, 2008) and photographed in a Tecnai electron microscope as described in the previous section. We examined randomly chosen BR mRNPs in the interchromatin space and docked at NPCs after double labeling and determined whether they were labeled or not.

A BR mRNP was considered labeled when an immunogold particle was on top of the BR mRNP or within 20 nm of the BR mRNP (estimated length of the primary plus secondary antibody). For the hrp45/SRp55 + 9G8 combination, a total of 865 BR mRNPs were analyzed. Of these, 11% were labeled with the hrp45/SRp55 antibody, 11% were labeled with the 9G8 antibodies, and 2.3% of the BR mRNPs were labeled with both antibodies. For the SC35 + 9G8 combination, 908 BR mRNPs were analyzed. 20% of the BR mRNPs were labeled with the anti-SC35 antibody, 8% were labeled with the 9G8 antibodies, and 3.2% were labeled with both antibodies.

For docked BR mRNPs, a total of 106 BR mRNPs were analyzed for the hrp45/SRp55 + 9G8 combination. 9% were labeled with the hrp45/SRp55 antibody, 11% were labeled with the 9G8 antibodies, and 2.8% were labeled with both antibodies. For the SC35 + 9G8 combination, a total of 133 BR mRNPs were analyzed. 15% were labeled with the SC35 antibody, 14% were labeled with the 9G8 antibodies, and 4% were labeled with both antibodies.

After staining with the anti-eIF4H antibodies (Björk et al., 2003), we obtained a value of 0.5% labeled BR mRNPs (the total number of examined BR mRNPs was 1,729). After staining with the anti–von Willebrand antibody or with only secondary antibody, the percentage of labeled BR mRNPs was even lower. Therefore, the immuno-EM labeling of BR mRNPs was ∼15–30 times higher with the anti–SR protein antibodies than the background labeling with the unspecific antibodies.

Analysis of protein–poly(A)$^+$ RNA association
Cytoplasm was purified from UV-irradiated *C. tentans* epithelial tissue culture cells by cell lysis and centrifugation, and poly(A)$^+$ RNA was isolated from cytoplasm using Oligotex (QIAGEN) according to the manufacturer's protocol. After elution of the cytoplasmic poly(A)$^+$ RNA and RNase treatment, the copurified proteins were analyzed by Western blotting.

Analysis of polysomes
C. tentans epithelial tissue culture cells were UV irradiated and homogenized in 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, and 2 mM DTT. The homogenate was centrifuged at 15,000 *g* for 10 min, and the supernatant was layered on top of a 15–50% sucrose gradient. After centrifugation at 150,000 *g* for 1.5 h at 4°C in a rotor (AH650; Sorvall), 100-µl fractions were collected, measured at A_{260nm}, and analyzed by Western blotting. EDTA was added to the homogenate to a final concentration of 30 mM where indicated (+EDTA).

Microinjection into the nucleus
Salivary glands were dissected from *C. tentans* fourth instar larvae and placed in a drop of ZO medium (Wyss, 1982) surrounded by paraffin oil. 2.5 µg/µl anti-SC35 antibody or 2.5 µg/µl of a control antibody was injected into individual nuclei (InjectMan NI2 [Eppendorf] and FemtoJet [Eppendorf] connected to an Axiovert 135 microscope [Carl Zeiss, Inc.]). Three to five cells per gland were injected with ∼0.01 nl of antibody solution per nucleus. Injected glands were incubated in hemolymph for 90 min at room temperature. After washing in PBS, the glands were fixed in PBS containing 2% glutaraldehyde and 50 mM sucrose for 2 h at 4°C. The glands were then washed in PBS containing 50 mM sucrose overnight at 4°C, dehydrated in ethanol, embedded in Agar 100 mixture, and prepared for EM as described for isolated chromosomes in the previous section. In each experiment, injected cells were analyzed and compared with uninjected cells and/or cells injected with a control antibody from the same salivary gland. Three types of analyses were performed.

The distribution of BR pre-mRNPs along the BR genes was analyzed. In the sections through active BR gene loci, short, random regions of the genes are visible. They were classified as proximal, middle, or distal segments based on the morphology of the BR pre-mRNPs using the criteria described in Immunofluorescence analyses and immuno-EM on isolated chromosomes. The area of each gene region was measured, and the relationships between the total areas for the proximal, middle, and distal segments were calculated. These areas reflect the amount of BR pre-mRNPs present on each gene segment and measure the relative distribution of the pre-mRNPs in the three segments of the gene.

Three independent experiments were performed for each treatment: uninjected, injected with monoclonal IgG antibody, and injected with anti-SC35 antibody. In each experiment, the analyzed gene regions comprised ∼10 complete BR genes (corresponding to ∼1,000 BR pre-mRNPs).

In the interchromatin space, BR mRNPs were counted in 10 randomly chosen areas (each area = 1.5 × 1.5 µm) in cells injected with anti-SC35 antibody (three independent experiments with a total of 315 BR mRNPs counted and a total of 171 BR mRNPs in the uninjected reference cells) and in control cells injected with anti–Ct-RSF antibodies (two independent experiments with a total of 492 BR mRNPs counted and a total of 498 BR mRNPs in the uninjected reference cells).

For each experiment, both in the case of BR mRNPs in the interchromatin space and docked BR mRNPs (see next paragraph), the ratio between the number of BR mRNPs in the injected cell and in an uninjected control cell in the same gland was calculated to take into account that different individuals exhibit slightly different levels of expression of the BR genes. For statistical analyses, *t* test was performed on log-transformed data.

The number of BR mRNPs docked at NPCs was counted along 60 µm (mean distance analyzed per cell) of nuclear membrane in cells injected with anti-SC35 antibody (three independent experiments with a total of 107 BR mRNPs counted and a total of 64 BR mRNPs in the uninjected reference cells) and in cells injected with anti–Ct-RSF antibodies (two independent experiments with a total of 71 BR mRNPs counted and a total of 66 BR mRNPs in the uninjected reference cells). The BR mRNPs located within 50 nm from the nuclear membrane were classified as docked to NPCs (Soop et al., 2005).

Online supplemental material
Fig. S1 shows the *C. tentans* SR proteins and the specificity of the anti–SR protein antibodies. Fig. S2 shows the primary structure of the *C. tentans*

ASF/SF2, 9G8, and SC35 proteins in comparison with the human and *D. melanogaster* homologues. Fig. S3 shows the reproducibility of staining of polytene chromosomes with anti-SC35 antibody. Fig. S4 shows ASF/SF2 staining of chromosome I and IV and staining of 9G8 and hrp45/SRp55 in heat shock gene loci. Fig. S5 shows the association of SR proteins with BR mRNPs at NPCs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200806156/DC1.

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