Specific combinations of SR proteins associate with single endogenous pre-mRNAs in vivo and individual SR proteins contribute different functions during gene expression

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ABSTRACT

SR proteins are required for constitutive and alternative splicing and influence the fate of mRNAs by being involved in export, mRNA surveillance and translation. It is unknown which SR proteins associate with a particular endogenous pre-mRNP/mRNP and if individual SR proteins are responsible for specific functions during gene expression *in vivo*.

We analyze the association of four SR proteins: ASF/SF2, SC35, 9G8 and hrp45 (SRp55/B52) with 125 nascent transcripts in *Chironomus tentans*. All transcripts associate with multiple types of SR proteins, in specific combinations. ASF/SF2, SC35 and 9G8 associate with the Balbiani ring (BR) pre-mRNPs early during transcription in agreement with a role in splicing. In contrast, hrp45 continuously associates with the BR pre-mRNPs during transcription, suggesting a function in addition to splicing. All four SR proteins remain with the BR mRNPs during export to the cytoplasm. Interference with SC35 function indicates that SC35 is important for release of the BR mRNP from the gene, and for export through the nuclear pore complex. ASF/SF2 is associated with polyribosomes, while SC35, 9G8 and hrp45 are associated with monoribosomes.

Individual endogenous pre-mRNPs/mRNPs thus bind multiple types of SR proteins and these SR proteins play different roles during gene expression *in vivo*.

INTRODUCTION

Within the superfamily of proteins containing an arginine- and serine-rich (RS) domain, a set of ten proteins, the SR proteins, is conserved in metazoans (reviewed by: Graveley 2000; Sanford et al. 2003). They share a similar domain structure, consisting of one or two N-terminal RNA-binding domains and a C-terminal RS-domain. The SR proteins are essential splicing factors *in vitro* and have redundant functions, since any individual SR protein can restore splicing activity in an S100 splicing deficient extract (Fu et al. 1992; Mayeda et al. 1992; Zahler et al. 1992). It is likely that individual SR proteins also have non-redundant functions (Singh and Valcárcel 2005). SR proteins bind specific RNA sequences (for references, 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 (Kim et al. 1992; 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 vivo, SRp55/B52 is essential in *Drosophila melanogaster* (*D. melanogaster*) (Ring and Lis 1994) and ASF/SF2 is essential in chicken B-cells (Wang et al. 1996) and in *Caenorhabditis elegans* (*C. elegans*) (Longman et al. 2000). In *C. elegans*, several other SR proteins are not essential for survival, but knock down of multiple SR proteins is lethal (Longman et al. 2000). Knockout in mice of SRp20 (Jumaa et al. 1999), SC35 (Wang et al. 2001; Xiao et al. 2007) or ASF/SF2 (Xu et al. 2005) leads to embryonal lethality.

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, the SR proteins, bound to exonic splicing enhancers, ESEs, participate in a network of protein-protein interactions through their RS domains. These protein interactions promote splicing in several different ways (Graveley 2000). Secondly, RS domains play essential roles by directly contacting splice site signals (Shen and Green 2004; Shen and Green 2006; Shen and Green 2007). The phosphorylated RS domains presumably bind splicing signals that are partially base-paired with U snRNAs and promote this base-pairing. Multiple RS domain-RNA interactions, including the branchpoint and the 5′ splice site, are required for splicing.

SR proteins are important for alternative splicing (Graveley 2000). They can influence alternative exon inclusion by recruiting U2AF to weak 3′ splice sites or by antagonizing hnRNP proteins bound to splicing silencer sequences. The presence of SR protein binding sites inside introns may result in inhibition of splicing (Kanopka et al. 1998). Expression of SR proteins can differ between different tissues (Zahler et al. 1993; Screaton et al. 1995: Hoffman and Lis 2000), and the balance between SR proteins and hnRNPs may differ in different tissues (Hanamura et al. 1998). Individual SR proteins can have antagonistic influence on splice site selection (Gallego et al. 1997; Jumaa and Nielsen 1997), but it is not known to what extent individual SR proteins are responsible for tissue specific alternative splicing. Alternative splicing might be decided by which combination of splicing factors, including SR proteins, that associates with a pre-mRNA. Differential recruitment of SR proteins to alternatively spliced transcripts supports such a combinatorial model (Mabon and Misteli 2005).

SR proteins are important for other steps in mRNA formation and transport. Some SR proteins shuttle between the nucleus and the cytoplasm (Cáceres et al. 1998) and facilitate mRNA transport. SRp20 and 9G8 are involved in export of intronless mRNAs (Huang and Steitz 2001), and SRp20, 9G8 and ASF/SF2 are adapters for TAP/NXF1-dependent mRNA export (Huang et al. 2003; Lai and Tarn 2004). ASF/SF2 regulates mRNA stability (Lemaire et al. 2002) and stimulates translation (Sanford et al., 2004). In addition, SR proteins are involved in 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. Further, SRp55/B52 in *D. 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 if an individual SR protein can perform all the different SR protein functions during splicing and further steps of biogenesis of individual transcripts, or if several different types of SR proteins must act together during expression of a gene. Since 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. On the other hand, experimental data suggest that multiple independent SR proteins take part in excision of each intron (Graveley 2000; Shen and Green 2004). Most experiments that are relevant for this issue have been performed *in vitro* or used artificial gene constructs. It is therefore important to obtain information on which SR proteins that associate with endogenous genes transcripts, when they do so and when they leave the transcripts.

Here, 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* (*C. tentans*) to study the association of individual SR proteins with gene specific pre-mRNPs and mRNPs *in vivo.* We show that four individual SR proteins, ASF/SF2, 9G8, SC35 and hrp45, bind to nascent pre-mRNAs in a gene specific combination. The BR pre-mRNPs bind all four SR proteins co-transcriptionally, but the distribution of the individual SR proteins along the nascent pre-mRNP is different. The four SR proteins then remain associated with the BR mRNPs during transport and nucleo-cytoplasmic export. In the cytoplasm, ASF/SF2 remains associated with the mRNA in polyribosomes, while 9G8, SC35 and hrp45 are restricted to 80S complexes. Our data suggest that different types of SR proteins have different roles during expression of specific genes.

RESULTS

SR proteins associate in specific combinations with nascent pre-mRNAs

We wished to obtain an overall view of the association of SR proteins with nascent pre-mRNPs. We therefore stained polytene chromosomes from *C. tentans* salivary gland cells with four SR protein specific antibodies. Antibodies specific for ASF/SF2, 9G8, SC35 and hrp45 were obtained and tested for their specificity (see Materials and Methods and Supplemental Material, Fig. S1). The SR protein hrp45 is the *C. tentans* homologue to *D. melanogaster* B52 and mammalian SRp55. In Fig. 1, we show chromosome I, immunolabeled for two SR proteins at a time, 9G8 plus hrp45, or 9G8 plus SC35. We show staining of ASF/SF2 alone, because we could not technically perform double staining with this antibody. The immunosignals were abolished when the chromosomes were pre-treated with RNase (data not shown), suggesting that the association of the SR proteins with gene loci was dependent on the presence of nascent pre-mRNA. In chromosome I, we recorded approximately 40 well stained loci with the anti-9G8 (Fig. 1A), anti-hrp45 (Fig. 1B), anti-SC35 (Fig. 1E) and anti-ASF/SF2 (Fig. 1G) antibodies respectively. Approximately the same number of loci was recorded on chromosomes II and III, while chromosome IV had about seven stained loci. In total, we recorded the association of the SR proteins with approximately 125 specific gene loci. The most striking result was the similarity in the binding pattern for all four SR proteins.

The relative intensity of staining for the four SR proteins varied in different gene loci. There were gene loci that, based on the staining intensities, contained relatively more 9G8 than hrp45 (arrow in Fig. 1A and B), and vice versa (arrow-head in Fig. 1A and B). The same was true when 9G8 was compared to SC35. Largely, the staining pattern for SC35 and hrp45 coincided (as compared to 9G8 and by comparing chromosomes stained separately for SC35 and hrp45). The locus indicated by the large arrowhead in Fig. 1A, B, D and E, was such an example. There were also differences between hrp45 and SC35. For example, there was relatively more SC35 and 9G8 in the locus marked by a line in Fig. 1A, B, D and E, while there was little if any hrp45 in this locus. For ASF/SF2, we detected largely the same staining pattern along chromosome I as for the other three SR proteins (Fig. 1G). Again, the relative staining intensity differed at some gene loci. Two loci (small arrowheads in Fig. 1A, B, D, E and G), contained relatively much ASF/SF2 as compared to other loci. In contrast, the locus marked with a star (Fig. 1A, B, D, E and G) contained much less ASF/SF2 compared to the other three SR proteins.

Apart from the BR genes (Wieslander 1994), only few other genes have been described in *C. tentans*, among them the heat shock genes (Sass 1995). We observed differential binding of SR proteins to heat shock pre-mRNA (Fig. S4). In general, we observed that SC35 association with all heat shock pre-mRNAs was consistently much lower than that for 9G8 and hrp45.

On chromosome IV, the three BR gene loci, BR1, BR2 and BR3, contained substantial amounts of all four SR proteins (Fig. 2A-G); no obvious relative differences could be seen. Another gene locus contained 9G8, SC35 and ASF/SF2, while very little hrp45 could be detected (arrowhead in Fig. 2A, B, D, E and G).

In conclusion, our results show that, of the at least 125 gene specific nascent pre-mRNPs investigated, all bound multiple types of SR proteins. In each gene locus, we detected at least three of the four analysed

SR proteins and in about 90% of the loci, all four SR proteins were present. Some loci bound all four SR proteins approximately to the same extent, as was the case for the BR genes. Others bound little or nothing of one of the SR proteins. We also observed that the relative abundance of the SR proteins varied in different gene loci. It is therefore likely that each nascent transcript bind a specific combination of the four SR proteins.

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

The immunofluorescence results strongly suggested, but did not prove, that each individual transcript binds multiple SR proteins. To ascertain that this was the case, we investigated the association of the SR proteins with BR1 and BR2 pre-mRNPs at the electron microscope level. The BR1 and BR2 pre-mRNAs associate with various RNA binding proteins co-transcriptionally and gradually form morphologically characteristic pre-mRNA-protein complexes, referred to as BR pre-mRNPs. We analyzed the association of the SR proteins with the BR1 and BR2 genes by immuno-EM on isolated polytene chromosome IV. We found that the SR proteins became part of the growing BR pre-mRNPs on the transcribing gene. Growing BR pre-mRNPs labeled for each SR protein can be seen in Fig. 3A-D. All studied SR proteins were found associated with BR pre-mRNPs in all different stages of transcription and packaging.

To know if multiple types of SR proteins were simultaneously present in an individual BR pre-mRNP, we performed double labeling experiments. We found that an individual BR pre-mRNP was simultaneously associated with at least two SR proteins. In Fig. 3E-F, we show nascent BR pre-mRNPs simultaneously labeled with SC35 plus 9G8, and hrp45 plus 9G8. Based on our observation, we conclude that an individual BR pre-mRNA associates with more than one type of SR protein during transcription.

The SR proteins are differently distributed along the BR genes

To map the distribution of SR proteins along the transcribing BR genes, we studied the association of SR proteins with the nascent pre-mRNPs on the different segments of the BR genes by immuno-EM. Based on the morphology of the BR pre-mRNPs as described in Materials and Methods and as shown in Fig. 4A, it is possible to determine if a nascent BR pre-mRNP belongs to the 5′ proximal (p), middle (m) or 3′ distal (d) segment of the gene. Representative segments of the transcribing BR gene are shown in Fig. 4A, and a schematic representation of the entire gene loop based on reconstruction data (Daneholt el al. 1982) is shown in Fig. 4B.

We found that all four SR proteins were present in BR pre-mRNPs located in the proximal, middle and distal segments of the gene (Fig. 3A-D and Fig. 4C). For SC35, 9G8 and ASF/SF2 we found 50-60% of the gold labeling in the proximal segment, about 30% in the middle and 10-20% in the distal segments. In contrast, the hrp45 labeling increased along the gene in agreement with a previous observation (Alzhanova-Ericsson et al. 1996).

These results show that the SR proteins are present in the BR pre-mRNPs all along the transcribed gene and that they all associate with the BR pre-mRNPs early during transcription. The relative distribution of hrp45 shows that this protein is added to the pre-mRNP continuously during transcription. The opposite situation was seen for SC35, 9G8 and ASF/SF2, i.e. there was a net loss of these SR proteins during transcription.

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

BR mRNPs are morphologically distinct granular structures as they are present in the interchromatin (Daneholt 2001). We could therefore investigate by immuno-EM if the SR proteins remain bound to the BR mRNPs after release from the genes. We found that BR mRNPs present in the interchromatin were labeled with antibodies directed against all four SR proteins. In double labeling experiments we observed that two of the SR proteins were simultaneously present in individual BR mRNPs. The labeling for hrp45 plus 9G8, and for SC35 plus 9G8 are shown in Fig. 5A, A′, C and C′. We also analyzed BR mRNPs during translocation through the NPC. Double labeled BR mRNPs (hrp45 plus 9G8, SC35 plus 9G8) passing through the NPCs are shown in Fig. 5B, B′, D, and D′. We conclude, that multiple types of SR proteins remain associated with individual BR mRNPs during transport through the interchromatin and when the BR mRNPs were translocated through the NPCs to the cytoplasm.

SR proteins remain associated with mRNA in the cytoplasm

ASF/SF2, SC35, 9G8 and hrp45 were all found in the cytoplasm, as shown by nuclear-cytoplasmic fractionation and Western blotting (Fig. S5). Immuno-EM also showed significant labeling for the SR proteins in the cytoplasm (data not shown). SC35 in *C. tentans* and *D. melanogaster* lacks the nuclear retention sequence present at the C-terminus in mammalian SC35 (Fig. S3). This is in agreement with the fact that we found SC35 in the cytoplasm.

The cytoplasmic SR proteins were bound to mRNA as shown by UV cross-linking experiments (Fig. 6A). In the case of hrp45, this result was also supported by the observation that hrp45 was found associated with the cytoplasmic part of the BR mRNP during translocation through the NPC (Fig. 5B, B′).

To analyze if the SR proteins remain associated with the mRNA during translation, we fractionated cytoplasmic extracts from tissue culture cells on sucrose gradients and analyzed the different fractions for the presence of individual SR proteins by Western blotting. Only ASF/SF2 was associated with polyribosomes in an EDTA sensitive manner (Fig. 6B). SC35, 9G8 and hrp45 were detected in the 60-80S part of the gradient, but not in the polyribosome part (Fig. 6B). EDTA treatment of the extracts shifted SC35, 9G8 and hrp45 from the 60-80S to the 40-60S 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 all remain bound to mRNA in the cytoplasm and that ASF/SF2 was associated with mRNA during translation. SC35, 9G8 and hrp45 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 is important for release of BR pre-mRNPs from the gene

In order to analyze the importance of SC35 for BR mRNP biogenesis, we interfered with SC35 function *in vivo*. We injected the anti-SC35 antibodies into the cell nuclei of salivary gland cells and analysed the effect on synthesis of BR mRNPs. First, we investigated if the nascent BR pre-mRNPs were affected. We did not observe any significant change in the overall characteristics of the transcribing BR gene loops. The number and length of loops with nascent pre-mRNPs on the proximal, middle and distal segments of the active gene were not significantly altered. Thus we did not record any obvious influence on the transcription of the BR genes 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 hnRNPs and other proteins was not influenced at the level of resolution investigated.

Based on morphological criteria (see above), we analysed the distribution of pre-mRNPs on the different segments of a transcribing BR gene in detail. In 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 (Fig. 7). In the injected cells, the distribution of BR pre-mRNPs along the gene was altered (Fig. 7). The most significant difference was the increase in the relative number of almost complete transcripts in the distal segment of the gene and the decrease in the relative number of transcripts in the middle segment. This means that we recorded an overrepresentation of almost complete nascent BR pre-mRNPs at the 3′ end of the gene. In the EM sections it was not possible to directly measure the spacing of the pre-mRNPs, but a closer spacing of the pre-mRNPs in the distal segment is compatible with the observed morphology. We conclude that interfering with SC35 *in vivo,* influenced the relative distribution of transcripts along the BR gene, resulting in a relative accumulation of transcripts in the 3′ end distal segment of the gene. This suggests that SC35 is involved in processing events that are necessary for releasing the mRNP from the gene.

SC35 is important for BR mRNP transport *in vivo*

We analysed the export of the BR mRNPs in cells injected with the anti-SC35 antibody. In the preparations used, it was not possible to directly identify the BR mRNPs in transit through the NPC channels. However, we could identify the BR mRNPs docked at the basket of the NPC. Based on previous characterizations (Kiseleva et al. 1996; 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. We found that injection of anti-SC35 antibodies resulted in a significant increase of the number of docked mRNPs (Fig. 8A). Consistent with this finding, the number of BR mRNPs in the interchromatin space increased in the injected cells compared to the control cells (Fig. 8B). 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. This suggests that SC35 contributes to export of BR mRNPs *in vivo*.

DISCUSSION

We report data on the association of four different types of SR proteins with a large set of nascent pre-mRNPs transcribed from endogenous genes. We further describe the association of these SR proteins with nascent pre-mRNPs and mRNPs derived from the BR genes, whose exon-intron structure and splicing characteristics are known. We can therefore 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 SR proteins co-transcriptionally in gene specific combinations

More than 90% of the 125 gene loci contained ASF/SF2, 9G8, SC35 and hrp45, and all loci contained at least three of these SR proteins. As a rule, individual nascent pre-mRNPs therefore associates with multiple types of SR proteins. The SR proteins are recruited as a consequence of transcription as shown by the fact that heat shock gene loci stained only after heat induction. Transcription dependent recruitment of hrp45 has been demonstrated also for BR genes (Björk et al. 2006).

Approximately 10% of the analyzed gene loci contained obvious specific combinations of SR proteins. It is likely that the immunostaining did not reveal small amounts of SR proteins at a gene locus or small differences in abundance. Our data therefore suggest that although most gene loci contained all four SR proteins, there was a gene specific combination of the abundance of the proteins.

How can we explain that the pre-mRNPs associate with multiple types of SR proteins in specific combinations? It is unlikely that different expression of the SR proteins in the cells is the reason. ASF/SF2 is relatively less abundant in the salivary gland cells while the other three SR proteins are approximately equally abundant (data not shown). Since our analyses were made on genes active in the same cell, we assume that gene specific properties determined the SR protein association. SR proteins have substrate specificity defined by their RRM domain(s) (Mayeda et al. 1999). RNA sequence motifs with some SR protein binding specificity have also been identified (reviewed by 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. In addition, other proteins presumably influence binding of SR proteins.

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 their SR protein binding partners and 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 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 (e.g. Baurén and Wieslander 1994; Baurén et al. 1998; Wetterberg et al. 2001; Daneholt 2001). We can therefore uniquely relate the association of the different SR proteins to BR gene structure, transcription and processing *in vivo*. The BR1 and 2 genes have three introns within the first 3 kb of the gene and a fourth intron located downstream the 35 kb long exon 4, and about 600 bp from the polyadenylation site (Wieslander 1994). The BR3 gene contains 38 short introns evenly distributed in the 11 kb gene (Paulsson et al. 1990). The BR1, 2 and 3 genes encode secretory proteins. They are efficiently expressed and their pre-mRNAs are constitutively spliced. All four SR proteins associated extensively with BR1, 2 and 3 pre-mRNAs, regardless of the large difference in intron numbers and hence 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 (reviewed by Graveley 2000), and that more than one SR protein molecule contacts the pre-mRNA during splicing (Shen and Green 2004; Shen and Green 2006; Shen and Green 2007). *In vitro*, a single type of SR protein is sufficient for splicing. It is therefore conceivable that this is possible also *in vivo*.

We detected four different types of SR proteins as part of the BR1 and 2 pre-mRNPs very early during transcription, at the time when introns 1-3 appear in the transcript. These four SR proteins can, including hrp45 (Björk et al. 2006), support splicing *in vitro*. ASF/SF2, SC35 and 9G8 where relatively most abundant in the proximal 7 kb segment of the BR1 and 2 genes. This was especially obvious for ASF/SF2 (Fig. 4C). This correlates as to location and time with the co-transcriptional splicing of the three proximal introns (Baurén and Wieslander 1994). Interestingly, these three SR proteins then declined along the gene, indicating that the proteins to a considerable extent leave the BR pre-mRNP after completion of excision of introns 1-3. Substantial amounts of molecular mass are added to and lost from the transcription-splicing complex during co-transcriptional 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.

Not all SR proteins leave the pre-mRNP after excision of introns 1-3. Due to the fact that the middle segment of the BR 1 and 2 genes is three times as long as the proximal and distal segments, the relative distribution of the SR proteins shown in Fig. 4, underestimates the relative abundance of the SR proteins in the proximal and distal segments. All four SR proteins, and in particular hrp45 are therefore present when intron four excision is initiated. In summary, our data show that four types of SR proteins are present both when introns 1-3 are excised and when intron 4 is excised, suggesting that more than one type of SR protein is involved when single introns are excised *in vivo*.

The distribution of hrp45 along the BR pre-mRNPs suggests an additional role for the protein apart from splicing. A net increase in hrp45 abundance suggests that hrp45 binds at many sites along the BR pre-mRNP. This indicates that hrp45 is a repeated entity during folding/packaging of the pre-mRNP and therefore influences this process. Since this RNA is largely built from approximately 200 nucleotides long repeats (Wieslander 1994), it is possible that each repeat contains a binding site for hrp45.

Individual SR proteins have multiple roles during expression of individual genes

Our functional studies of SC35 show that individual SR proteins have several different functions in the cell nucleus during processing and export of a single mRNP. When we interfered with SC35 function, BR pre-mRNPs accumulated at the end of the gene, suggesting that transcription was unaffected but that release from the gene was slowed down. 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). A coupling between excision of the last intron and 3′ end cleavage and polyadenylation has been described also in other genes (for example Vagner et al. 2000; Kyburz et al. 2006; Rigo and Martinson 2008). Impairment of SC35 function during excision of intron 4 could therefore affect the release of the pre-mRNPs from the gene. The partial effect suggests that SC35 is important but not essential during excision of intron 4 and the coupling between splicing, transcription termination and 3′ end processing, although we can not rule out that SC35 function was only partially inhibited by the antibody.

An alternative mechanism could be that SC35 has a function analogous to the yeast SR protein-like Npl3p. This 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, where splicing of the transcript is essentially complete (Baurén and Wieslander 1994). BR mRNPs as well as other mRNPs (Singh et al. 1999; Politz et al. 1999; Shav-Tal et al. 2004), move by diffusion through the interchromatin and subsequently dock at the basket of the NPCs. We showed that interfering with SC35 partially inhibited export through the NPC, leading to a doubling of BR mRNPs in the nucleus. BR mRNPs are delivered from the genes to the interchromatin and then stochastically exported through the NPCs. A pool of BR mRNPs is therefore present in the interchromatin. This pool is approximately constant in size and completely turned over during 90-120 minutes (Singh et al. 1999). Based on these facts, we estimate that the block in export is partial and in the order of 20-25%. Based on previous analyses of BR mRNP export through NPCs (Kiseleva et al. 1996; 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 top of the basket.

Our data are compatible with a role for *C. tentans* SC35 as an export adapter, perhaps for the general mRNA export factor TAP/NXF1. This proposal is based on the experimental data showing a role for *C. tentans* SC35 in BR mRNP export and the fact that *C. tentans* SC35 lacks the nuclear retention sequence present in mammalian SC35 and that the protein is bound to mRNA in the cytoplasm. 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). Since ASF/SF2, 9G8 and hrp45 are also part of the BR mRNPs at the NPCs, it is possible that several SR proteins contribute to export of an individual BR mRNP.

In conclusion, SC35 is part of BR pre-mRNPs and mRNPs and the functional consequences of antibody interference demonstrates that SC35 plays at least two roles within the cell nucleus during expression of the BR genes.

Different association of SR proteins with mRNPs during translation

Our data show that the four different 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, consistent with a role during translation. In agreement with these data, ASF/SF2 is known to be associated with polyribosomes also in mammalian cells, where it has been shown to stimulate cap-dependent translation (Sanford et al. 2004).

Additional studies have 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 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 only in the 80S ribosome region (Fig. 6C). It appears that these three SR proteins dissociated from the mRNPs prior to cap-dependent translation in polyribosomes. It is therefore possible that these three SR proteins are involved in translation initiation or possibly in a first round of translation connected to NMD (Zhang and Krainer 2004). In either case, the different behavior compared to ASF/SF2 indicates differences in function for the different SR proteins.

MATERIALS AND METHODS

Biological material

Animals and cells

C. tentans was cultured as described (Meyer et al. 1983). A *C. tentans* embryonic epithelial cell line was cultured as described (Wyss 1982).

Antibodies

The monoclonal anti-SC35 and the polyclonal anti-9G8 antibodies were produced in our laboratory (Supplemental Material). Anti-ASF/SF2 monoclonal antibody (Hanamura et al. 1998) was a gift from A. Krainer or obtained from Zymed Laboratories. Monoclonal antibodies against hrp45 and hrp36 (Kiseleva et al. 1994; Wurtz et al. 1996) was a gift from B. Daneholt. The specificities of the anti-SR protein antibodies were checked by Western blotting, (see Fig. S1). The secondary antibodies used for Western blots were: swine anti-rabbit Ig HRP and goat anti-mouse Ig HRP (DakoCytomation), both diluted 1:3000. The secondary antibodies used for immunofluorescence analyses were: rabbit anti-mouse FITC Ig, swine anti-rabbit Ig FITC (DakoCytomation), donkey anti-mouse IgG Texas Red (Jackson Immuno Research), goat anti-rabbit IgG Cy5 and donkey anti-mouse IgG Cy5 (Amersham Pharmacia Biotech), all diluted 1:100. The secondary antibodies used in immunoelectron microscopy were: goat anti-mouse IgG 6 nm or 12 nm colloidal gold and goat anti-rabbit IgG 6 nm or 12 nm colloidal gold (Jackson Immuno Research).

Isolation of polytene chromosomes

Salivary glands from *C. tentans* fourth instar larvae were fixed in 2% paraformaldehyde in TKM (10 mM triethanolamine-HCl, pH 7.0, 100 mM KCl, and 1 mM $MgCl_2$) for 2 min at 4°C. Repeated pipetting through a glass micropipette released the chromosomes (Björk and Wieslander 2008). The chromosomes were transferred to a siliconized glass slide and fixed for 30 min in TKM containing 4% paraformaldehyde at room temperature. The preparations were processed for immunofluorescence or EM analyses as described below.

Immunofluorescence analyses on isolated chromosomes

After fixation of isolated chromosomes, immunofluorescence staining was performed as described (Björk and Wieslander 2008). Preparations were analyzed in a LSM 510 confocal microscope (Carl Zeiss).

Immunoelectron microscopy on isolated chromosomes

After fixation, isolated chromosomes were subjected to immunoreactions with primary and gold conjugated secondary antibodies. The chromosomes were post-fixed in 2% glutaraldehyde in TKM, dehydrated in ethanol and embedded in Agar 100 mixture as described (Björk and Wieslander 2008). The embedded chromosomes were sectioned and thin sections (70-80 nm) were collected on copper grids, stained with 2% uranyl acetate in 50% ethanol for 10 min and washed in 50% ethanol. The specimens were photographed in a FEI 120 kV Tecnai electron microscope (at 80 kV) using a Gatan US 1000P CCD camera.

Immunoelectron microscopy on cryosections

Preparations of ultrathin cryosections were performed as described (Visa et al., 1996). In brief, salivary glands were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min at room temperature. After rinsing, the glands were cryoprotected with 2.3 M sucrose and frozen in liquid nitrogen. Ultra thin cryosections (70-80 nm) were deposited onto nickel grids coated with formvar and carbon. The grids were blocked on drops of 0.1 M glycine in PBS, containing 10% fetal calf serum for 20 min, and incubated with the primary antibody for 60 min, and subsequently with the secondary antibody conjugated with 6 or 12 nm gold particles (Jackson ImmunoResearch) for 60 min at room temperature. The sections were stained with 2% aqueous uranyl acetate and embedded in polyvinyl alcohol (9-10 kD, Sigma-Aldrich). The specimens were photographed in a FEI Tecnai electron microscope as above.

Analysis of protein-poly(A+) RNA association

C. tentans tissue culture cells were UV-irradiated and poly(A+) RNA was isolated from cytoplasm using Oligotex according to the manufacturer's protocol (Qiagen). After elution of poly(A+) RNA and RNase treatment, the co-purified proteins were analyzed by Western blotting.

Analysis of polysomes

C. tentans tissue culture cells were UV-irradiated and homogenized in 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT. The homogenate was centrifuged at 15.000xg for 10 min and the supernatant was layered on top of a 15 to 50% sucrose gradient. After centrifugation at 150.000xg for 1.5 hours at +4°C in a Sorvall AH650 rotor, 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).

Western blotting

Proteins to be analyzed by Western blotting were boiled in sample buffer (62.5 mM Tris-HCl, pH 6.8 containing 10% glycerol, 2.3% SDS, 5% mercaptoethanol and 0.02% bromophenol blue) and separated on 12% SDS-PAGE. The separated proteins were transferred to PVDF-filters by semi-dry electrophoresis. HRP-labeled secondary antibodies were detected by the ECL method (Amersham Pharmacia Biotech).

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. Anti-SC35 antibody (2.5 µg/µl) or a control antibody, was injected into individual nuclei (Eppendorf InjectMan NI2 and FemtoJet connected to a Zeiss Axiovert 135 microscope). 3-5 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 2h at +4°C. The glands were then washed in PBS containing 50 mM sucrose over night at +4°C, dehydrated in ethanol, embedded in Agar 100 mixture and prepared for electron microscopy as described above for isolated chromosomes.

In each experiment, injected cells were analyzed and compared with non-injected cells or cells injected with a control antibody, from the same salivary gland. Three types of analyses were done.
The number of BR mRNPs in the interchromatin space was counted in ten randomly chosen areas (each area 1.5 x 1.5 μm) in control cells and in injected cells. Data was collected from three independent experiments.

The number of BR mRNPs docked at NPCs was counted along 60 μm (average distance analyzed per cell) of nuclear membrane in control cells and in injected cells. Data was collected from three independent experiments. The BR mRNPs located within 50 nm from the inner surface of the nuclear membrane were classified as docked to NPCs (Kiseleva et al. 1996; Soop et al. 2005).

The distribution of BR pre-mRNPs along the BR genes was analyzed. The morphology of the BR pre-mRNPs is different in the proximal (20% of the gene), middle (60% of the gene) and distal (20% of the gene) segments of the BR1 and 2 genes (see Fig. 4A, B). In the sections through active BR gene loci, short, random parts of the genes are visible and identified based on the pre-mRNP morphology. The BR pre-mRNPs in the EM sections were classified into proximal, middle and distal pre-mRNPs according to their morphology and the relative proportion of each class of pre-mRNP was calculated. These proportions are a measure of the relative distribution of the pre-mRNPs in the three segments of the gene. In control cells, the pre-mRNPs are evenly spaced along the gene and the recorded proportions (20%, 60%, 20%) agreed with the relative lengths of the three gene segments.

In injected cells and control cells, the analyzed BR pre-mRNPs comprised approximately 10 complete BR genes (approximately 1000 BR pre-mRNPs) respectively. Data was collected from two independent experiments.

Analyses of SR proteins along an active BR gene

The SR proteins were localized on the BR gene using the specific antibodies and secondary antibodies, conjugated with gold particles. The distribution of SR proteins along the BR gene was determined by assigning each gold particle to one of the three segments of the gene, the proximal, middle and distal segments. The percentage of gold particles in each segment was calculated.

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REFERENCES

Alzhanova-Ericsson, A.T., Sun, X., Visa, N., Kiseleva, E., Wurtz, T., and Daneholt, B. 1996. A protein of the SR family of splicing factors binds extensively to exonic Balbiani ring pre-mRNA and accompanies the RNA from the gene to the nuclear pore. *Genes & Dev.* **10:** 2881-2893.

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

Baurén, G., Belikov, S., and Wieslander, L. 1998. Transcriptional termination in the Balbiani ring 1 gene is closely coupled to 3′-end formation and excision of the 3′-terminal intron. *Genes & Dev.* **12:** 2759-2769.

Bedard, K.M., Daijogo, S., and Semler, B.L. 2007. A nucleo-cytoplasmic SR protein functions in viral IRES-mediated translation initiation. *EMBO J.* **26:** 459-467.

Björk, P., Wetterberg-Strandh, I., Baurén, G. and Wieslander, L. 2006. Chironomus tentans-repressor splicing factor represses SR protein function locally on pre-mRNA exons and is displaced at correct splice sites. *Mol. Biol. Cell* **17:** 32-42.

Björk, P., and Wieslander, L. 2008. Gene expression in polytene nuclei. in *The Nucleus, Methods in Molecular Biology*, vol. 2 (ed. R. Hancock), Humana Press. In press.

Cáceres, J.F., Screaton, G.R., and Krainer, A.R. 1998. A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. *Genes & Dev.* **12:** 55-66.

Calzone, F.J., Angerer, R.C,. and Gorovsky, M.A. 1982. Regulation of protein synthesis in Tetrahymena: isolation and characterization of polysomes by gel filtration and precipitation at pH 5.3. *Nucleic Acids Res.* **10:** 2145-2161.

Cavaloc, Y., Popielarz, M., Fuchs, J.P., Gattoni, R. and Stevenin, J. 1994. Characterization and cloning of the human splicing factor 9G8: A novel 35 kDa factor of the serine/arginine protein family. *EMBO J.* **13:** 2639-2649.

Daneholt, B., Andersson, K., Björkroth, B., and Lamb, M.M. 1982. Visualization of active 75 S RNA genes in the Balbiani rings of Chironomus tentans. *Eur. J. Cell. Biol.* **26:** 325-332.

Daneholt, B. 2001. Assembly and transport of a premessenger RNP particle. *Proc. Natl. Acad. Sci.* **98:** 7012–7017.

Das, R., Yu, J., Zhang, Z., Gygi, M.P., Krainer, A.R., Gygi, S.P., and Reed, R. 2007. SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol. Cell* **26:** 867-881.

Fu, X.-D. 1993. Specific commitment of different pre-mRNAs to splicing by single SR proteins. *Nature* **365:** 82-85.

Fu, X.-D., Mayeda, A., Maniatis, T., and Krainer, A. R. 1992. General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5' and 3' splice site selection. *Proc. Natl. Acad. Sci.* **89:** 11224-11228.

Gallego, M.E., Gattoni, R., Stevenin, J., Marie, J., and Expert-Bezancon, A. 1997. The SR splicing factors ASF/SF2 and SC35 have antagonistic effects on intronic enhancer-dependent splicing of the beta-tropomyosin alternative exon 6A. *EMBO J.* **16:** 1772-1784.

Gilbert, W., and Guthrie, C. 2004. The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA. *Mol. Cell* **13:** 201-212.

Graveley, B.R. 2000. Sorting out the complexity of SR protein functions. *RNA* **6:** 1197-1211.

Hanamura, A., Caceres, J.F., Mayeda, A., Franza JR., B.R., and Krainer, A.R. 1998. Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA* **4:** 430-444.

Hoffman, B.E., and Lis, J.T. 2000. Pre-mRNA splicing by the essential *Drosophila* protein B52: tissue and target specificity. *Mol. Cell. Biol.* **20:** 181-186.

Huang, Y., and Steitz, J.A. 2001. Splicing factrors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. *Mol. Cell* **7:** 899-905.

Huang, Y., Gattoni, R., Stévenin, J., and Steitz, J.A. 2003. SR splicing factors serve as adapter proteins for TAP-dependent mRNA export. *Mol. Cell* **11:** 837-843.

Jumaa, H., and Nielsen, P.J. 1997. The splicing factor SRp20 modifies splicing of its own mRNA and ASF/SF2 antagonizes this regulation. *EMBO J.* **16:** 5077-5085.

Jumaa, H., Wei, G., and Nielsen, P.J. 1999. Blastocyst formation is blocked in mouse embryos lacking the splicing factor SRp20. *Curr. Biol.* **9:** 899-902.

Kanopka, A., Mühlemann, O., Petersen-Mart, S., Estmer, C., Öhrmalm, C., and Akusjärvi, G. 1998. Regulation of adenovirus alternative RNA splicing by dephospohorylation of SR proteins. *Nature* **393:** 185-187.

Kim, Y.J., Zou, J.L., Manley, J.L., and Baker, B.S. 1992. The *Drosophila* RNA-binding protein RBP1 is localized to transcriptionally active sites of chromosomes and shows a functional similarity to human splicing factor ASF/SF2. *Genes & Dev.* **6:** 2569-2579.

Kiseleva, E., Wurtz, T., Visa, N., and B. Daneholt. 1994. Assembly and disassembly of spliceosomes along a specific pre-messenger RNP fiber. *EMBO J.* **13:** 6052-6061.

Kiseleva, E., Goldberg, M.W., Daneholt, B., and Allen T.D. 1996. RNP export is mediated by structural reorganization of the nuclear pore basket. *J. Mol. Biol.* **260:** 304-311.

Kyburz, A., Friedlein, A., Langen, H., and Keller, W. 2006. Direct interaction between subunits of CPSF and the U2 snRNP contribute to the coupling of pre-mRNA 3′ end processing and splicing. *Mol. Cell* **23:** 195-205.

Lai, M.-C., and Tarn, W.-Y. 2004. Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins. *J. Biol. Chem.* **279:** 31745-31749.

Lemaire, R., Prasad, J., Kashima, T., Gustafson, J., Manley, J.L., and Lafyatis, R. 2002. Stability of a PKCI-1 related mRNA is controlled by the splicing factor ASF/SF2: A novel function for SR proteins. *Genes & Dev.* **16:** 594-607.

Li, X., and Manley, J. L. 2005. Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* **122:** 365–378.

Li, X., Wang, J., and Manley, J. L. 2005. Loss of splicing factor ASF/SF2 induces G2 cell cycle arrest and apoptosis, but inhibits internucleosomal DNA fragmentation. *Genes & Dev.* **19:** 2705-2714.

Liu, H.-X., Zhang, M.. and Krainer, A.R. 1998. Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. *Genes & Dev.* **12:** 1998-2012.

Liu, K. J., and Harland, M. H. 2005. Inhibition of neurogenesis by SRp38, a neuroD-regulated RNA binding protein. *Development* **132:** 1511-1523.

Longman, D., Johnstone, I.L., and Cáceres, J.F. 2000. Functional characterization of SR and SR-related genes in *Caenorhabditis elegans*. *EMBO J.* **19:** 1625-1637.

Mabon, S.A., and Misteli, T. 2005. Differential recruitment of pre-mRNA splicing factors to alternatively spliced transcripts in vivo. *Plos Biol.* **3:** 1893-1901.

Mayeda, A., Zahler, A.M., Krainer, A.R., and Roth, M.B. 1992. Two members of a conserved family of nuclear phosphoproteins are involved in pre-mRNA splicing. *Proc. Natl. Acad. Sci.* **89:** 1301-1304.

Mayeda, A., Screaton, G.R., Chandler, S.D., Fu, X.-D., and Krainer, A.R. 1999. Substrate specificities of SR proteins in constitutive splicing are determined by their RNA recognition motifs and composite pre-mRNA exonic elements. *Mol. Cell. Biol.* **19:** 1853-1863.

Meyer, B., Mähr, R., Eppenberger H.-M., and Lezzi, M. 1983. The activity of Balbiani rings 1 and 2 in salivary glands of *Chironomus tentans* larvae under different modes of development and after pilocarpine treatment. *Develop. Biol.* **98:** 265-277.

Misteli, T., Caceres, J.F., Clement, J.Q., Krainer, A.R., Wilkinson, M.F., and Spector, D.L. 1998. Serine phosphorylation of Sr proteins is required for their recruitment to sites of transcription in vivo. *J. Cell Biol.* **143:** 297-307.

Paulsson, G., Lendahl, U., Galli, J., Eriksson, E., and Wieslander, L. 1990. The Balbiani ring 3 gene in *Chironomus tentans* has a diverged repetitive structure split by many introns. *J. Mol. Biol.* **211:** 331-249.

Phair, R.R.d and Misteli, T. 2000. High mobility of proteins in the mammalian cell nucleus. *Nature* **404:** 604-609.

Politz, J.C., Tuft, R.A., Pederson, T., and Singer, R.H. 1999. Movement of nuclear poly(A) RNA throughout the interchromatin space in living cells. *Curr. Biol.* **9:** 285-291.

Rasheva, V. I., Knight, D., Bozko, P., Marsh, K., and Frolov, M. V. 2006. Specific role of the SR protein splicing factor B52 in cell cycle control in Drosophila. *Mol. Cell. Biol*. **26:** 3468-3477.

Rigo, F., and Martinson, H.G. 2008. Functional coupling of last-intron splicing and 3'-end processing to transcription in vitro: the poly(A) signal couples to splicing before committing to cleavage. *Mol. Cell. Biol.* **28:** 849-862.

Ring, H.Z., and Lis, J.T. 1994. The SR protein B52/SRp55 is essential for Drosophila development. *Mol. Cell. Biol.* **14:** 7499-7506.

Sanford, J.R., Longman, D., and Cáceres, J.F. 2003. Multiple roles of the SR protein family in splicing regulation. *Progr. Molec. Subcell. Biology* **31:** 33-58.

Sanford, J.R., Gray, N.K., Beckman, K., and Cáceres, J.F. 2004. A novel role for shuttling SR proteins in mRNA translation. *Genes & Dev.* **18:** 755-768.

Sass, H. 1995. Transcription of heat shock gene loci versus non-heat shock loci in Chironomus polytene chromosomes: evidence for heat-induced formation of novel putative ribonucleoprotein particles (hsRNPs) in the major heat shock puffs. *Chromosoma* **103:** 528-538.

Sato, H., Hosoda, N., and Maquat, L.E. 2008. Efficiency of the pioneer round of translation affects the cellular site of nonsense-mediated mRNA decay. *Mol. Cell 29:* 255-262.

Schaal, T.D., and Maniatis, T. 1999. Multiple distinct splicing enhancers in the protein-coding sequences of a constitutively spliced pre-mRNA. *Mol. Cell Biol.* **19:** 261-273.

Screaton, G.R., Cáceres, J.F., Mayeda, A., Bell, M.V., Plebanski, M., Jackson,D.G., Bell, J.I., and Krainer, A.R. 1995. Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. *EMBO J.* **14:** 4336-4349.

Shav-Tal, Y., Darzacq, X., Shenoy, S.M., Fusco, D., Janicki, S.M., Spector, D.L., and Singer, R.H. 2004. Dynamics of single mRNPs in nuclei of living cells. *Science* **304:** 1797-1800.

Shen, H., and Green, M.R. 2004. A pathway of sequential arginine-serine-rich domain-splicing signal interactions during mammalian spliceosome assembly. *Mol. Cell* **16:** 363-373.

Shen, H., and Green, M.R. 2006. RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans. *Genes & Dev.* **20:** 1755-1765.

Shen, H., and Green, M.R. 2007. RS domain-splicing signal interactions in splicing of U12-type and U2-type introns. *Nature Struct. & Mol. Biol.* **14:** 597-603.

Shin, C., and Manley, J.L. 2002. The SR protein SRp38 represses splicing in M phase cells. *Cell* **111:** 407-417.

Shin, C., Feng, Y., and Manley, J.L. 2004. Dephosphorylated SRp38 acts as a splicing repressor in response to heat shock. *Nature* **427:** 553-558.

Singh, O.P., Björkroth, B., Masich, S., Wieslander, L., and Daneholt, B. 1999. The intranuclear movement of Balbiani ring premessenger ribonucleoprotein particles. *Exp. Cell Res.* **251:** 135-146.

Singh, R., Valcárcel, J. 2005. Building specificity with nonspecific RNA-binding proteins. *Nature Struct. Mol. Biol.* **12:** 645-653.

Soop. T., Ivarsson, B., Björkroth, B., Fomproix, N., Masich, S., Cordes, V.C., and Daneholt, B. 2005. Nup153 affetcs entry of messenger and ribosomal ribonucleoproteins into the nuclear basket during export. *Mol. Biol. Cell* **16:** 5610-5620.

Sun, X., Alzhanova-Ericsson, A. T., Visa, N., Aissouni, Y., Zhao, J., and Daneholt, B. 1998. The hrp23 protein in the Balbiani ring pre-mRNA particles is released just before or at the binding of the particles to the nuclear pore complex. *J. Cell Biol.* **142:** 1181–1193.

Swartz, J.E., Bor, Y.-C., Misawa, Y., Rekosh, D., and Hammarskjöld, M.-L. 2007. The shuttling SR protein 9G8 plays a role in translation of unspliced mRNA containing a constitutive transport element. *J. Biol. Chem.* **282:** 19844-19853.

Tacke, R., and Manley, J.L. 1999. Determinants of SR protein specificity. *Curr. Opin. Cell Biol.* **11:** 358-362.

Vagner, S., Vagner, C., and Mattaj, I.W. 2000. The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF 65 to couple 3′-end processing and splicing. *Genes & Dev.* **14:** 403-413.

Visa, N., Alzhanova-Ericsson, A.T., Sun, X., Kiseleva, E., Björkroth, B., and Daneholt, B. 1996. A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell* **84:** 253-264.

Wang, J., and Manley, J.L. 1995. Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways. *RNA* **1:** 335-346.

Wang, J., Takagaki, Y., and Manley, J.L. 1996. Targeted disruption of an essential vertebrate gene: ASF/SF2 is required for cell viability. *Genes & Dev.* **10:** 2588-2599.

Wang, H.Y., Xu, X., Ding, J.H., Mermingham, J.R. Jr., and Fu, X.D. 2001. SC35 plays a role in T cell development and alternative splicing of CD45. *Mol. Cell* **7:** 331-342.

Wetterberg, I., Zhao, J., Masich, S., Wieslander, L., and Skoglund, U. 2001. In situ transcription and splicing in the Balbiani ring 3 gene. *EMBO J.* **20:** 2564-2574.

Wieslander, L. 1994. The Balbiani ring multigene family: Coding repetitive sequences and evolution of a tissue specific cell function. *Progr. Nucl. Acid. Res. Mol. Biol.* **48:** 275-313.

Windgassen, M., Sturm, D., Cajigas, I.J., González, C.I., Seedorf, M., Bastians, H., and Krebber, H. 2004. Yeast shuttling proteins Npl3p, Gbp2p, and Hrb1p are part of the translating mRNPs, and Np13p can function as a translational repressor. *Mol. Cell. Biol.* **24:** 10479-10491.

Wurtz, T., Kiseleva, E., Nacheva, G., Alzhanova-Ericsson, A.T., Rosén, A., and Daneholt, B. 1996. Identification of two RNA-binding proteins in Balbiani ring premessenger ribonucleoprotein granules and presence of these proteins in specific subsets of heterogeneous nuclear ribonucleoprotein particles. *Mol. Cell. Biol.* **16:** 1425–1435.

Wyss, C. 1982. *Chironomus tentans* epithelial cell lines sensitive to ecdysteroids, juvenile hormone, insulin and heat shock. *Exp. Cell Res.* **139:** 297-307.

Xiao, R., Sun, Y., Ding, J.-H., Lin, S., Rose, D.W., Rosenfeld, M.G., Fu, X.-D., and Li, X. 2007. Splicing regulator SC35 is essential for genomic stability and cell proliferation during mammalian organogenesis. *Mol. Cell. Biol.* **27:** 5393-5402.

Xu, X., Yang, D., Ding, J.H., Wang, W., Chu, P.H., Dalton, N.D., Wang, H.Y., Bermingham, J.R. Jr., Ye, Z., Liu, F. et al. 2005. ASF/SF2-regulated CaMKII alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell* **120:** 59-72.

Zahler, A.M., Lane, W.S., Stolk, J.A., and Roth, M.B. 1992. SR proteins: A conserved family of pre-mRNA splicing factors. *Genes & Dev.* **6:** 837-847.

Zahler, A.M., Neugebauer, K.M., Lane, W.S., and Roth, M.B. 1993. Distinct functions of SR proteins in alternative pre-mRNA splicing. *Science* **260:** 219-222.

Zahler, A.M., and Roth, M.B. 1995. Distinct functions of SR proteins in recruitment of U1 small nuclear ribonucleoprotein to alternative 5′splice sites. *Proc. Natl. Acad. Sci.* **92:** 2642-2646.

Zhang, Z., and Krainer, A. R. 2004. Involvement of SR proteins in mRNA surveillance. *Mol. Cell* **6:** 597-607.

FIGURE LEGENDS

Figure 1. Different types of SR proteins are associated with nascent pre-mRNAs.

Chromosome I was immuno-stained with combinations of anti-9G8 and anti-hrp45 antibodies (A-C) or anti-9G8 and anti-SC35 antibodies (D-F). Anti-ASF/SF2 antibodies were detected by silver enhancement of a gold-labeled secondary antibody (G). The large arrowhead indicates a gene locus where hrp45 and SC35 were enriched compared to 9G8. Small arrowheads indicate gene loci where all four SR proteins were present, but where the relative concentration of ASF/SF2 was especially high. The star indicates a gene locus that contained all four SR proteins, but relatively less of ASF/SF2. The arrow shows a gene locus that bound 9G8 and some SC35, but very little hrp45. The line shows a gene locus that was rich in 9G8 and SC35, but bound much less hrp45.

The size bar represents 20 μm.

Figure 2. Different types of SR proteins are associated with BR gene loci. Chromosome IV was immuno-stained with combinations of anti-9G8 and anti-hrp45 antibodies (A-C) or anti-9G8 and anti-SC35 antibodies (D-F). Staining with anti-ASF/SF2 antibodies is shown in G. The active BR1, BR2 and BR3

gene loci are indicated. The arrow shows a fourth gene locus, containing 9G8 and SC35, but little hrp45 and ASF/SF2. The size bar represents 20 μm.

Figure 3. Different types of SR proteins are associated with BR pre-mRNPs.

Chromosome IV was stained with anti-SC35 (A), anti-ASF/SF2 (B), anti-hrp45 (C) or anti-9G8 (D) antibodies and analysed by EM. Gold particles attached to the secondary antibodies show specific labeling of nascent BR pre-mRNPs (arrows). Combinations of anti-SC35 (6 nm gold) and anti-9G8 antibodies (12 nm gold) (E) and anti-hrp45 (6 nm gold) and anti-9G8 antibodies (12 nm gold) (F), showed that individual BR pre-mRNPs were associated with these combinations of SR proteins. E′ and F′ are schematic representations of double-labeled individual BR pre-mRNPs. The size bar shown in D represents 200 nm and applies to A-D. The size bar in F represents 100 nm and applies to E and F.

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 antibodies. For each SR protein, all gold particles were attributed to proximal (p), middle (m) or distal (d) segments of the BR gene. In A, a part of the BR gene locus is shown and examples of proximal, middle and distal gene segments are indicated. In B, an active BR gene with the growing BR pre-mRNPs is shown schematically. The three segments of the active gene are indicated. In C, the relative distribution of gold-labeling for each SR protein in each segment of the BR gene is presented. The size bar represents 200 nm.

Figure 5. Different types of SR proteins are associated with BR mRNPs during nucleo-cytoplasmic transport.

Sections through salivary gland nuclei were stained with combinations of anti-hrp45 and anti-9G8 antibodies (A, B) or with anti-SC35 and anti-9G8 antibodies (C, D). Examples of BR mRNPs associated with hrp45 (12 nm gold) and 9G8 (6 nm gold) or with SC35 (12 nm gold) and 9G8 (6 nm gold) in the interchromatin space are shown in A and C. Examples of BR mRNPs associated with hrp45 and 9G8 or SC35 and 9G8 during translocation through NPCs are shown in B and D. Schematic interpretations of the micrographs are shown in A′, B′, C′ and D′. The size bar represents 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.

SC35, 9G8, ASF/SF2 and hrp45 co-purified with 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 (A). ASF/SF2 is associated with polyribosomes, while 9G8, SC35 and hrp45 are associated 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 80S monoribosomes (B).

Fractions from the sucrose gradients were pooled as indicated and subjected to Western blot analyses for detection of the indicated proteins C).

Figure 7. SC35 influences release of BR mRNPs from the gene.

To interfere with SC35 function *in vivo*, anti-SC35 antibodies were injected into the nuclei of salivary gland cells. Non-injected cells in the same gland served as control cells. The glands were prepared for EM and the distribution of BR pre-mRNPs along the BR gene was analysed. The gene segments were analysed and classified as proximal, middle or distal segments based on the morphology of the nascent BR pre-mRNPs. Control cells are represented by white bars and injected cells by gray bars.

Figure 8. SC35 is involved in export of BR mRNPs.

The export of BR mRNPs was analysed in cells injected with the anti-SC35 antibody. BR mRNPs docked at NPCs (white arrow heads in A) were recorded. The scale bars represent 100 nm, (N; nucleus and C; cytoplasm). The number of docked BR mRNPs per μm of membrane was counted in injected cells and in control cells and the ratio of the two values are presented in the diagram.

The number of BR mRNPs per area (μm^2) of interchromatin was counted in injected cells and in control cells and the ratio of the two values are presented in the diagram in B.

Supplemental Material

FIGURES

Figure S1. SR protein specific antibodies.

The specificities of anti-hrp45, anti-ASF/SF2, anti-9G8 and anti-SC35 antibodies were tested in a Western blot. Nuclear extract from *C. tentans* tissue culture cells was probed with the indicated antibodies. Each antibody detected a single protein.

Figure S2. SR proteins present in *C. tentans* tissue culture cells. SR proteins were purified by a two step salt-precipitation method (Roth et al. 1991; Zahler et al. 1992) and separated on a polyacrylamide gel. Coomassie staining of the gel (Coomassie), showed a number of bands that was analysed by mass spectrometry. The four *C. tentans* SR proteins, hrp45, ASF/SF2, 9G8 and SC35 were identified. The purified SR proteins were recognized by the SR protein specific monoclonal antibody mAb104, as shown in the Western blot analyses (mAb104). M shows size markers.

Figure S3. Conservation of *C. tentans* SR proteins.

Sequence alignments of ASF/SF2, 9G8 and SC35 from *C. tentans* (ct), *D. melanogaster* (dm) and *H. sapiens* (hs) show a high degree of conservation. Amino acid residues labeled in black and grey are identical and similar respectively. Accession numbers for C. *tentans* ASF/SF2, 9G8 and SC35 are respectively. The nuclear retention sequence in human SC35 extends from position 192 to 221. Accession numbers: AM950284 (9G8), AM950285 (ASF/SF2), AM950286 (SC35).

Figure S4. SR proteins are associated with heat shock gene loci.

C. tentans larvae were heat shocked and polytene chromosomes were isolated from salivary gland cells. Immunostaining experiments showed that 9G8 (A, D) and hrp45 (B) are both associated with nascent pre-mRNA at locus 5C on chromosome IV, while SC35 (E) is not detected at this locus. The size bar represents 20 μm.

Figure S5. The SR proteins are present in the cytoplasm.

Nuclear (N) and cytoplasmic (C) extracts were prepared from *C. tentans* tissue culture cells. The presence of ASF/SF2, SC35, hrp45 and 9G8 in the two fractions were analysed by Western blot. All four SR proteins were detected in both the nucleoplasm and the cytoplasm, although to different extent.

MATERIALS AND METHODS

Biological material

Animals and cells: For heat shock treatment, *C. tentans* larvae were kept at 37°C for 60 minutes.

Antibodies: SR proteins were purified from *C. tentans* (Fig. S2) 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). Polyclonal antibodies were raised in rabbits. The 9G8 specific antibodies were purified by chromatography on CNBr-activated Sepharose 4B columns (Amersham Pharmacia Biotech) to which 9G8 had been coupled. The mAb104 antibody (Roth et al. 1990; Zahler et al. 1992) was a gift from M.B. Roth.

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* 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* lambda Zap cDNA library.

C. tentans 9G8 was found in a two-hybrid screen using the Ct-RSF/hrp23 protein as bait (Matchmaker Two-Hybrid System, Clontech). All sequencing reactions were performed with the DYEnamic ET Terminator Cycling Sequencing premix kit (Amersham Pharmacia Biotech) and analyzed on a 373A Automated DNA Sequencer (Applied Biosystems). The sequences of DNA and proteins were analyzed by programs in the GCG package (Devereaux et al. 1984) and the Biology Workbench package (http://workbench.sdsc.edu).

Protein preparation, Western blotting and sequence analysis: SR proteins from *C. tentans* tissue culture cells (Fig. S2) were prepared as described (Zahler et al.1992). Nuclear and cytoplasmic extracts of *C. tentans* tissue culture cells were prepared as described (Wurtz et al. 1996). Proteins to be analyzed by Western blotting were boiled in sample buffer and separated on 12% SDS-polyacrylamide gels. The separated proteins were transferred to PVDF-filters by semi-dry electrophoresis. HRP-labeled secondary antibodies were detected by the ECL method (Amersham Pharmacia Biotech).

For amino acid sequence analyzes, the gel piece containing the protein of interest was excised from the Coomassie stained gel and 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, 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) was allowed to adsorb into the gel pieces. After an overnight incubation, the reaction was stopped by acidification with TFA. Generated peptides were extracted and subsequently isolated by microbore reversed phase liquid chromatography using a SMART System (Amersham Pharmacia Biotech) on a 1x150 mm Kromasil C18 column from Column Engineering. Individual peptides were analyzed by Edman degradation on a 494 amino acid sequencer (Applied Biosystems) following the manufacturer's instruction.

References

Devereaux, J., Haeberli, P., and Smithies. O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12:** 387-395.

Hellman, U. 2000. Sample preparation by SDS/PAGE and in-gel digestions. In: *Proteomics in functional genomics. Protein Structure analysis.* (eds. Jollès, P., Jörnvall, H.) pp. 88:43-54 Birkhauser Verlag AG, Basel.

Roth, M.B., Murphy, C., and Gall, J.G. 1990. A monoclonal antibody that recognizes a phosporylated epitope stains lampbrush chromosome loops and small granules in the amphibian germinal vesicle. *J. Cell Biol.* **111:** 2217-2223.

Roth, M.B., Zahler, A.M., and Stolk, J.A. 1991. A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. *J. Cell Biol.* **115:** 587-596.

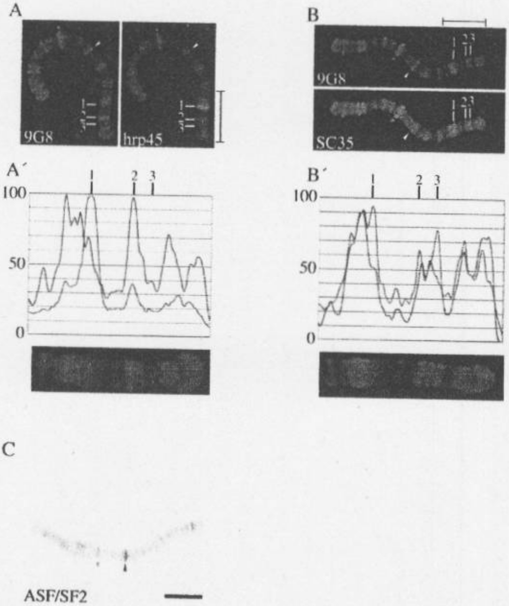

A
B
9G8
hrp45
9G8
SC35
A´
B´
1
2
3
100
50
0
C
ASF/SF2

Fig2

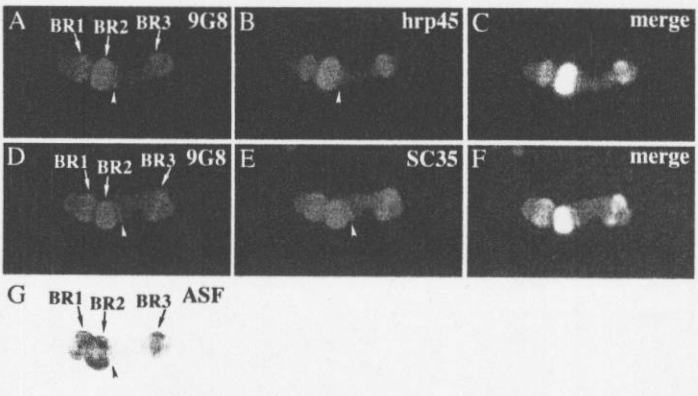

A
BR1
BR2
BR3
9G8
B
hrp45
C
merge
D
BR1
BR2
BR3
9G8
E
SC35
F
merge
G
BR1
BR2
BR3
ASF

Fig3

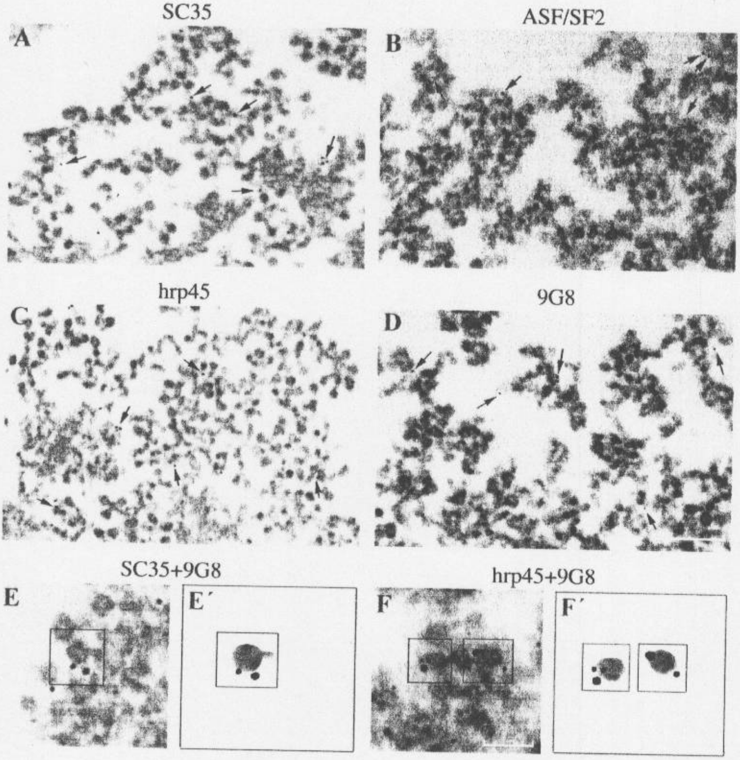


Fig4

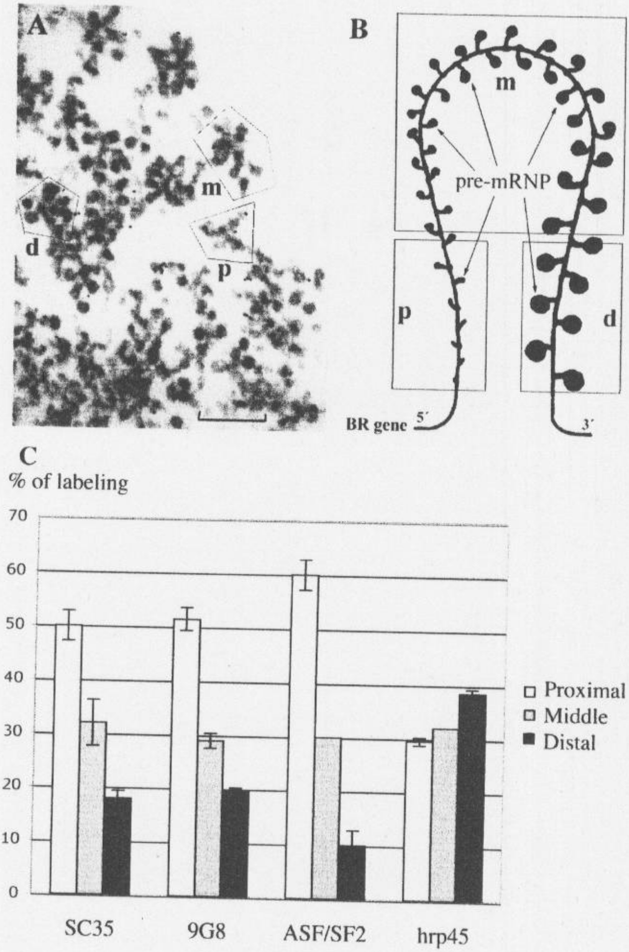
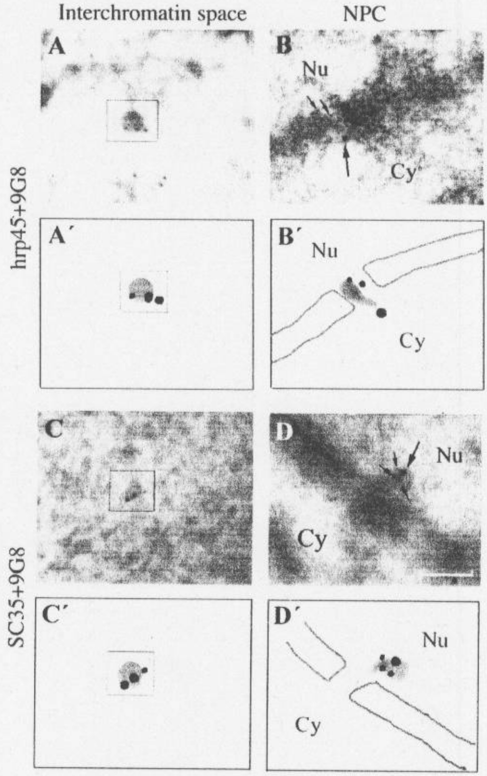

A
m
d
p
B
m
pre-mRNP
p
d
BR gene
5´
3´
C
% of labeling
70
60
50
40
30
20
10
0
SC35
9G8
ASF/SF2
hrp45
Proximal
Middle
Distal

Fig5



Interchromatin space
NPC
hrp45+9G8
SC35+9G8
A
B
Nu
Cy
A´
B´
Nu
Cy
C
D
Nu
Cy
C´
D´
Nu
Cy

Fig6

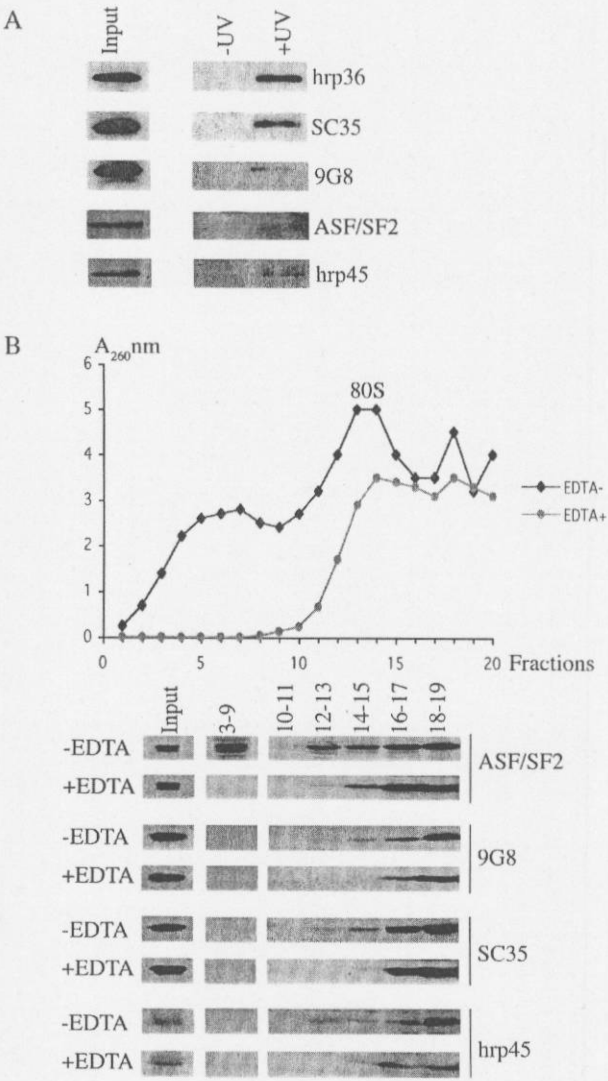

A
Input
-UV
+UV
hrp36
SC35
9G8
ASF/SF2
hrp45
B
A260nm
80S
EDTA-
EDTA+
Fractions
Input
3-9
10-11
12-13
14-15
16-17
18-19
-EDTA
+EDTA
ASF/SF2
9G8
SC35
hrp45

Fig7

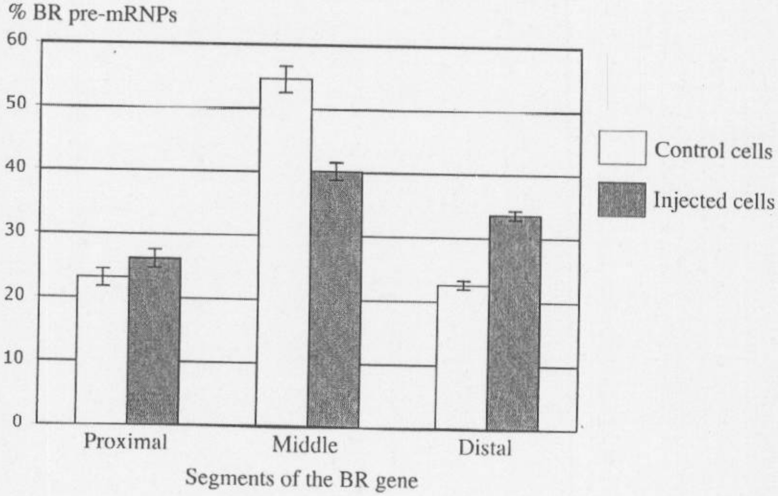
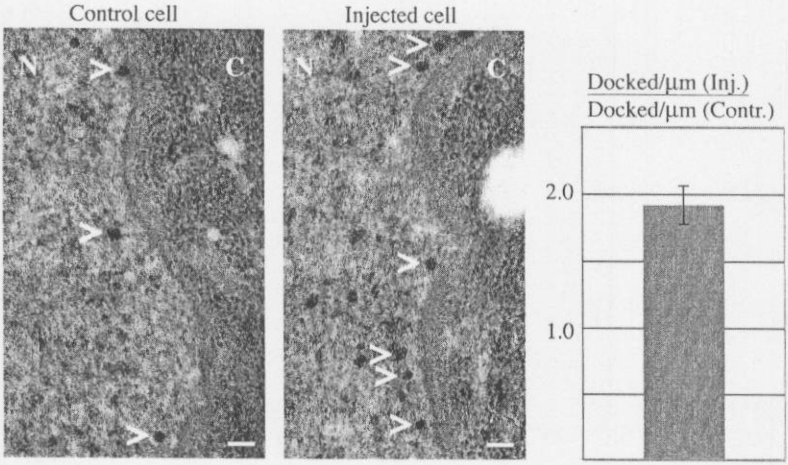

% BR pre-mRNPs
60
50
40
30
20
10
0
Control cells
Injected cells
Proximal
Middle
Distal
Segments of the BR gene

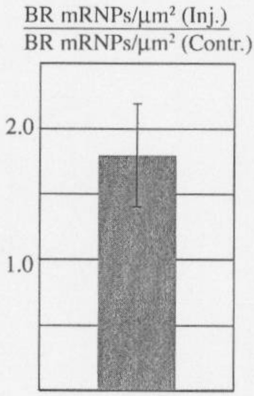
Fig8

A



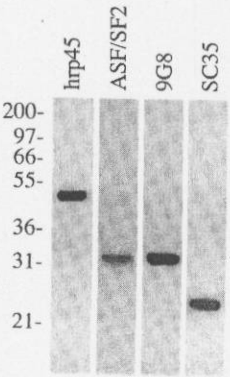
Control cell
Injected cell
N
C
N
C
Docked/μm (Inj.)
Docked/μm (Contr.)
2.0
1.0

B

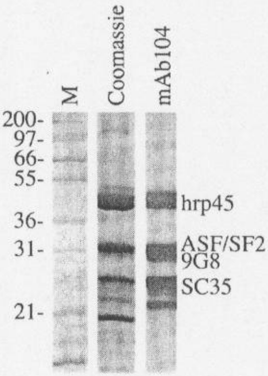


BR mRNPs/μm² (Inj.)
BR mRNPs/μm² (Contr.)
2.0
1.0

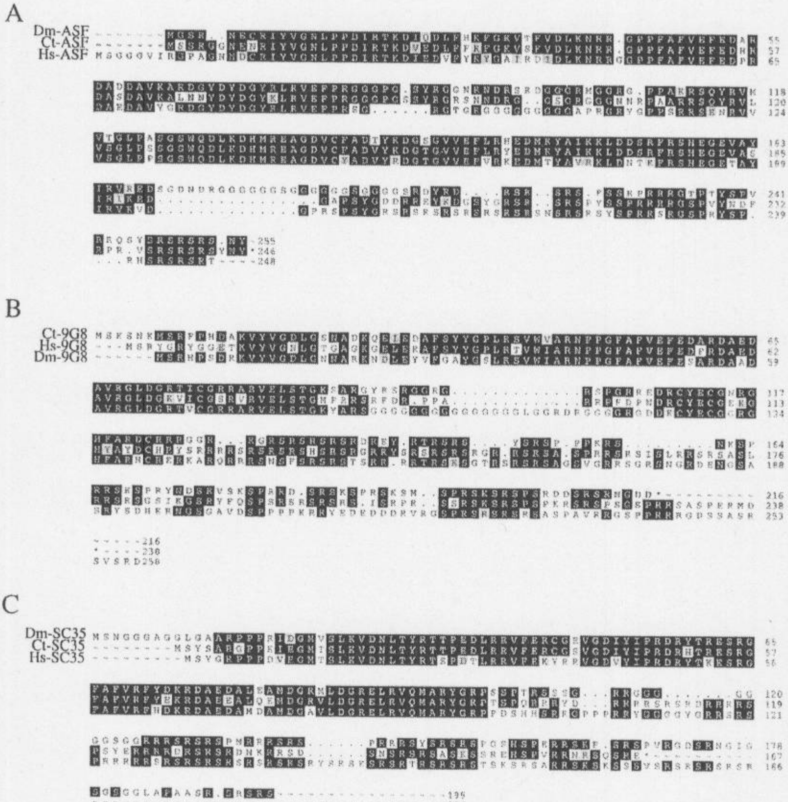
SupplementaryFigS1



SupplementaryFigS2



SupplementaryFigS3



SupplementaryFigS4