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SAF-B protein couples transcription and pre-mRNA splicing to SAR/MAR elements

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ABSTRACT

Interphase chromatin is arranged into topologically separated domains comprising gene expression and replication units through genomic sequence elements, so-called MAR or SAR regions (for matrix- or scaffold-associating regions). S/MAR regions are located near the boundaries of actively transcribed genes and were shown to influence their activity. We show that scaffold attachment factor B (SAF-B), which specifically binds to S/MAR regions, interacts with RNA polymerase II (RNA pol II) and a subset of serine-/arginine-rich RNA processing factors (SR proteins). SAF-B localized to the nucleus in a speckled pattern that coincided with the distribution of the SR protein SC35. Furthermore, we show that overexpressed SAF-B induced an increase of the 10S splice product using an E1A reporter gene and repressed the activity of an S/MAR flanked CAT reporter gene construct *in vivo*. This indicates an association of SAF-B with SR proteins and components of the transcription machinery. Our results describe the coupling of a chromatin organizing S/MAR element with transcription and pre-mRNA processing components and we propose that SAF-B serves as a molecular base to assemble a 'transcriptosome complex' in the vicinity of actively transcribed genes.

INTRODUCTION

Gene expression is regulated by transcriptional (1) and post-transcriptional events such as capping (2), RNA transport (3), RNA degradation (4,5) and pre-mRNA splicing (6,7). Biochemical and genetic analysis identified a multitude of proteins whose diverse roles and molecular interactions are currently being investigated. However, immunocytochemical studies revealed that a considerable number of those proteins co-localize in a characteristic speckled distribution within the nucleus. The observed speckles correspond to interchromatin granules and perichromatin fibrils observed by electron microscopy (8–10). Ultrastructural analysis of actively transcribed genes performed in *Drosophila* and mammalian cells (11,12) suggest that transcription and pre-mRNA splicing are temporally and spatially linked and that splicing factors are recruited to the sites of intron-containing RNA transcription (13). Based on these and other studies it is currently believed that splicing factors are stored and reassembled in interchromatin granule clusters, whereas RNA transcription and splicing occurs in perichromatin fibrils (14). Furthermore, it was shown that the shuttling of splicing factors between these sites was sensitive to kinase and phosphatase inhibitors (15). On a molecular level, several recent reports support the existence of large macromolecular complexes containing RNA polymerase II, capping-, splice- and polyadenylation factors (16–18). This supports the idea that RNA synthesis and maturation occur simultaneously in an 'RNA processing unit' (19).

Eukaryotic DNA, the substrate of this 'RNA factory', is organized in a highly ordered DNA–protein complex known as chromatin (20). It was shown that chemical modifications such as histone acetylation and DNA methylation regulate gene expression (21–23). In addition, several protein complexes harbouring chromatin restructuring activities influence gene expression in response to cell physiological changes (24).

Chromatin is thought to be organized into topologically separated loops comprising gene expression units. These domains are defined by DNA elements, so-called scaffold or matrix attachment regions (S/MAR regions), which usually comprise AT-rich sequences of high unwinding propensity (25,26). S/MAR elements can affect gene expression, as shown from experiments using heterologous promotors flanked by S/MAR elements, and they significantly lower position-dependent effects of stably integrated transgenes (27). Several S/MAR DNA binding proteins have been isolated and they are thought to mediate the attachment of chromatin to nuclear protein structures (28). Furthermore, S/MAR domains have been implicated in tissue-specific gene expression exemplified by the immunoglobulin μ chain expression in B lymphocytes (29). SATB1, which is

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predominantly expressed in thymocytes, indicates that tissue-specific S/MAR binding proteins may constitute an additional level to regulate gene expression (30). However, apart from their DNA binding properties, very little is known about their molecular characteristics.

We used yeast two-hybrid screens to look for novel proteins involved in either splicing or transcription processes and used the splice factor SRp30c, the C-terminal domain (CTD) of RNA pol II and the SR protein kinase CLK2 as baits. These screens identified a common interacting protein, scaffold attachment factor-B (SAF-B). In this report we investigated the molecular interactions of SAF-B and show that it formed a ternary complex with S/MAR DNA and SR proteins *in vitro*. Furthermore, we show that SAF-B was highly concentrated in nuclear speckles, where it co-localized with the splice factor SC35. Using overexpression systems we used splicing and CAT reporter gene assays to confirm the observed molecular interactions *in vivo*. Based on these experiments we propose a model in which SAF-B binds to S/MAR regions and forms a molecular assembly point to allow the formation of a 'transcriptosomal' complex consisting of SR proteins and RNA polymerase II.

MATERIALS AND METHODS

Cloning and library screening

Cloning of yeast two-hybrid baits was performed as described (31). Briefly, SRp30c cDNA was subcloned into pGBT9 (Clontech). Using this clone as a bait, an embryonic day E16 rat brain library in pADGal4 (Stratagene) was screened. From this screen 47 clones were analysed further, of which one clone was rSAF-B-PC. Other clones found in this screen corresponded to ribosomal proteins and a previously identified P-E-rich protein (32). The screen with the CTD-pol II is described elsewhere (33). Out of 36 positive clones isolated after screening 2 million transformants, 13 clones were different isolates of human SAF-B cDNA. CLK2 was cloned in pBMT116 and after screening 200 000 clones using the E16 rat brain library, five clones contained rSAF-B-PC.

Gelshift analysis

[α-^{32}P]dATP-labelled MII S/MAR PCR fragment or pUC18 PCR fragment (50 ng) were incubated with 100 ng GST-SAF-Δ*Xho* and increasing amounts of cold competitor DNA in HNTG (34). After 10 min, 5% glycerol was added and the DNA was separated on 1.2% TBE–agarose gels. Gels were dried and analysed by autoradiography.

Kinase assay and phosphoamino acid analysis

mCLK2 and mCLK2KR were overexpressed in A293 cells and lysed as described earlier (34). Immunoprecipitation with anti-mCLK2 antibodies was carried out overnight. The immunoprecipitate was washed three times with HNTG and once with kinase assay buffer (34). The kinase reaction was carried out with 1 µg of purified p43 at 30°C for 30 min. Following SDS–PAGE, the labelled proteins were transferred to PVDF membranes and visualized by autoradiography. The radioactive band corresponding to p43 was excised, hydrolysed and analysed by two dimensional chromatography on cellulose TLC plates as described (35).

Pull down and precipitation experiments

An aliquot of 5 µg of GST fusion protein (GST-ASF/SF2, GST-htra2-beta1, GST-SRp30c, GST) were incubated with [^{35}S]methionine-labelled *in vitro* transcribed/translated rSAF-B-PC (Promega) and GSH–Sepharose beads (Pharmacia) in HNTG buffer. After an overnight incubation and three washes with HNTG, the proteins were subjected to SDS–PAGE and the gel was exposed on a Molecular Dynamics PhosphoImager.

For pull down experiments, His tagged p43 was incubated with 10 µl (100 µg) of nuclear extract (36) in the presence of 30 mM imidazole, 1 mM $MgCl_2$ and Ni–agarose beads (Qiagen). Washing was performed three times with HNTG buffer containing 20 mM imidazole. RNA polymerase II was detected by western blot with the ARNA-3 antibody (Progen, Heidelberg) and ECL (Amersham). For precipitation experiments, 500 ng of PCR amplified MII S/MAR DNA product were added to 20 µl of HeLa nuclear extract. After 15 min incubation on ice the precipitate was recovered by 30 min centrifugation (15 000 *g*), washed once with buffer D (36) and analysed by western blot.

mCLK2 and mock transfected 293 cells were lysed in RIPA buffer (0.01 M sodium phosphate, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 1 mM NaF, 4 mM sodium orthovanadate, 1 mM PMSF, pH 7.2). The supernatant of a 100 000 *g* spin was diluted 4-fold in RIPA rescue (0.01 M sodium phosphate, 1 mM NaF, 5 mM β-glycerolphosphate, 2 mM sodium orthovanadate, 20 mM NaCl, 1 mM DTT, 1 mM PMSF, pH 7.2) and incubated with [^{35}S]methionine-labelled *in vitro* transcribed/translated rSAF-B-PC (Promega). Immunoprecipitations were carried out overnight with αCLK2 antibodies and the proteins were subsequently analysed on SDS–PAGE followed by autoradiography and ECL using αCLK2 antibodies.

FLAG-tagged rSAF-B-PC was transiently overexpressed in 293 cells. After lysis in RIPA buffer and a 4-fold dilution in RIPA rescue, immunoprecipitations were carried out with anti-FLAG antibodies (Santa Cruz) overnight. Western blots were done with the mAb104 (ATCC) and ECL (Amersham).

Immunofluorescence

rSAF-B-PC was subcloned into pEGFP-C2 (Clontech) thereby tagging rSAF-B-PC at its N-terminus with green fluorescent protein. HEK293 cells were transfected with 1 µg pEGFP-rSAF-B-PC using the $CaCl_2$ method (34). Gene expression and localization was analysed by confocal microscopy (Leica) as described (31).

Transfection experiments

A293 cells (6×10^5) were transfected with a total of 4 µg DNA using the $CaCl_2$ method (34). For splicing assays RNA was isolated using RNeasy columns (Qiagen). Reverse transcription and PCR were carried out as described (31), including 0.05 µCi [α-^{32}P]dATP/µl in the PCR reaction. Product analysis was carried out on 8% polyacrylamide gels followed by autoradiography.

NIH3T3 cells (6×10^5) were transfected with lipofectamine (Gibco) using 1 µg of the reporter lysozyme 5′ MAR-promoter-CAT-5′ MAR (+MAR) or 1 µg of a control reporter containing flanking non-MAR sequences (–MAR) (27) and increasing amounts of rSAF-B-PC (cloned in pcDNA3). DNA amounts were adjusted to 1.5 µg with pcDNA3. Cells were harvested and CAT assays were performed according to the manufacturer's instructions (Boehringer).

RESULTS

SAF-B interacts with splicing factors and the CTD of RNA polymerase II in a yeast two-hybrid system

To identify protein–protein interactions within the spliceosome and to isolate yet uncharacterized splice factors, we screened a rat brain post-natal day 5 library using the SR protein SRp30c as a bait in the yeast two-hybrid system. One of the positive cDNA clones encoded a partial sequence, rSAF-B-PC, which was similar to the C-terminal part of the human nuclear protein hSAF-B.

To obtain the full length rat clone we performed 5′ RACE experiments using double stranded adaptor ligated cDNA. A schematic diagram of the complete clone is shown in Figure 1A. Due to the presence of an in frame stop codon in the 5′ end, the rat cDNA (DDBJ/EMBL/GenBank accession no. AF056324) is 58 amino acids shorter than the published human sequence (37). Another difference lies in an insert (amino acids 209–228 of the rat sequence) composed of 14 glutamic acids and two glutamine residues in the rat protein (Fig. 1A). We speculate that these observed sequence variations may be due to alternative splicing. Both rat and human sequences contain a highly conserved region rich in glutamic acid/arginine repeats (amino acids 585–658, 36% E; 24% R, 92% identity), which was part of our yeast two-hybrid clone rSAF-B-PC. The presence of alternating positive and negative residues in this domain is reminiscent of similar dipeptide motifs found in phosphorylated RS domains of SR proteins and might be of particular significance for protein–protein interactions.

SAF-B was initially purified due to its specific binding to S/MAR DNA (38) and more recently it was isolated as a DNA binding protein displaying a repressive activity on the HSP27 promotor in human breast cancer cells (37). It was speculated that this promotor element could be part of an S/MAR element. These results strongly suggest that hSAF-B is a DNA binding protein with the potential to repress gene activity, possibly mediated by S/MAR elements. As purified hSAF-B was shown to aggregate in presence of MII S/MAR DNA, we applied gel retardation experiments and observed that our recombinant rSAF-B-PC fragment precipitated and retained the radioactive DNA fragments at the top of the gel. To further demonstrate specificity we employed competition experiments and showed that MII/rSAF-B-PC complexes were not competed by unlabelled non-specific pUC DNA, whereas pUC/rSAF-B-PC complexes were efficiently competed by unlabelled competitor MII S/MAR DNA (Fig. 1B). This indicated that the MII DNA binding properties of rat and human SAF-B were very similar and that the activity was localized to the C-terminal domain of the SAF-B protein.

Interestingly, in a parallel screen (33) that was aimed to find novel proteins which would interact with the CTD of the largest subunit of RNA polymerase II (CTD-pol II), we also isolated hSAF-B. This suggested that SAF-B might not only interact with members of the splicing machinery, but also with a component of the transcription apparatus.

Analysis of protein domains required for binding of SAF-B with splicing proteins and RNA pol II

We next tested rSAF-B-PC against several other SR or SR-like proteins in the yeast two-hybrid assay. As shown in Figure 2A, rSAF-B-PC interacts strongly with CTD-pol II (33), SRp30c (39), htra2-beta1 (31), ASF/SF2 (40,41) and mCLK2 (34).

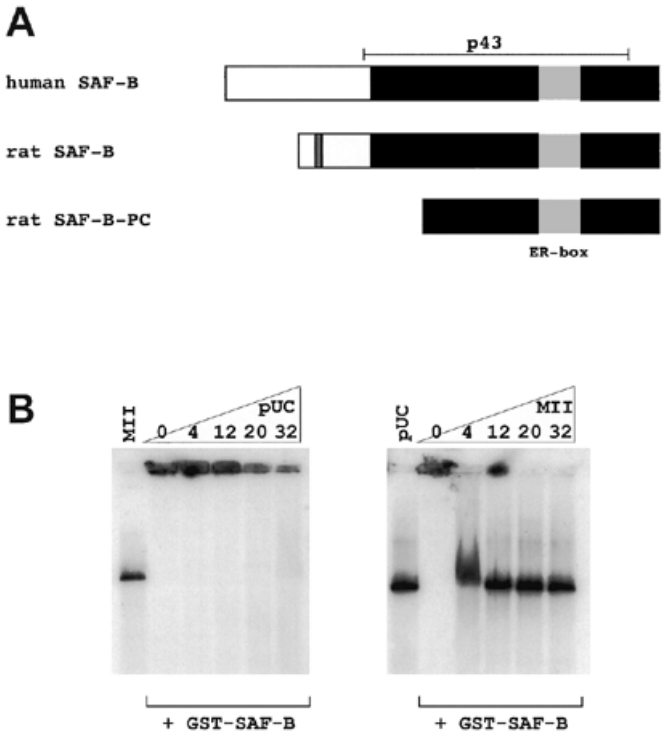

Figure 1. (**A**) Schematic diagram of the rat and human SAF-B proteins. The homologous C-terminal domains are drawn as black boxes, whereas the divergent N-terminal domains are coloured white. The E-rich insert of the rat protein is indicated as a dark shaded box; the ER-rich domain which is common to human and rat proteins is highlighted in grey. The p43 protein that was used in some experiments is indicated by a line. (**B**) Preferential binding of rSAF-B-PC to MII S/MAR DNA *in vitro*. GST-rSAF-BΔ*Xho*I was incubated with [α-^{32}P]dATP-labelled MII S/MAR DNA and increasing amounts of cold competitor pUC18 (left). Labelled pUC18 DNA was competed with excess cold MII S/MAR DNA (right).

A weaker interaction can be seen with U2AF35 (42) and SRp55 (39), whereas no interactions were detected with other members of the SR protein family such as SC35 (43), SRp40, SRp75 (39) and the Sm protein SmN (44). To elucidate the molecular basis of the SAF-B interaction, we constructed several C-terminal rSAF-B-PC deletion mutants and re-introduced them in the yeast two-hybrid system to assess their interaction with SRp30c, ASF/SF2, htra2-beta1, mCLK2 and CTD-pol II. We thereby determined that the minimal binding domain of rSAF-B-PC spans a fragment corresponding to amino acids 493–759 of the full length rat clone. Finally, deletion of the SR-domain of ASF/SF2 and SRp30c abolished binding to rSAF-B-PC in yeast (Fig. 2B).

SAF-B interacts with SR proteins and RNA pol II *in vitro* and *in vivo*

To verify the observed yeast two-hybrid interactions we used several methods to demonstrate a specific interaction of rSAF-B-PC with selected SR proteins and RNA pol II. To that end rSAF-B-PC was expressed and labelled with [^{35}S]methionine *in vitro* using rabbit reticulocyte lysates and tested for binding with purified recombinant GST tagged ASF/SF2, SRp30c, htra2-beta1 proteins or GST alone. All the investigated fusion proteins, but

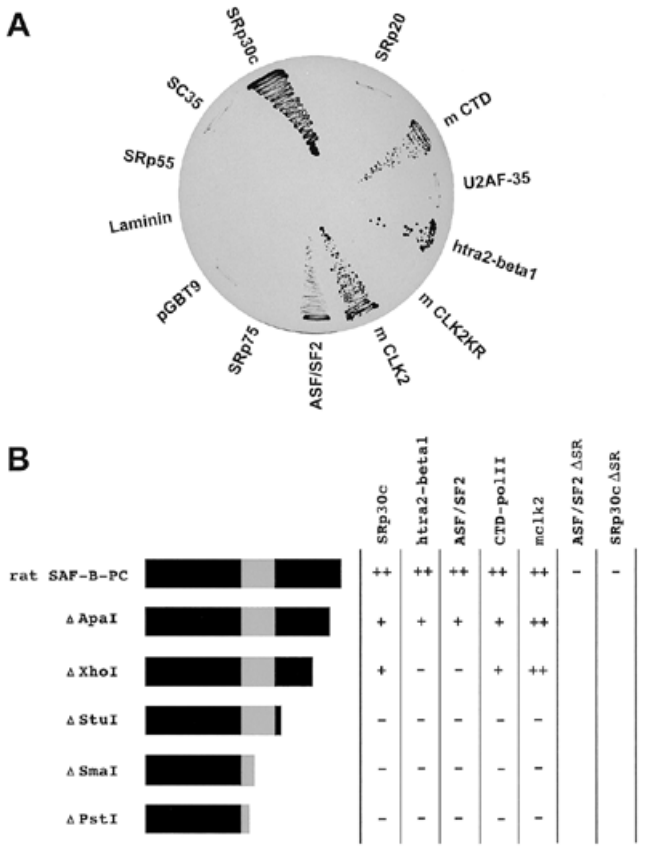

Figure 2. (**A**) Interaction of rSAF-B-PC with splicing factors in yeast. The interaction was tested against SC35, SRp30c, SRp55, SRp75, htra2-beta-1, SmN, SRp40, SF2/ASF, U2AF-35 and pGBT9 with laminin as negative control. Transformants were obtained on plates without 3-aminotriazole and then restreaked on plates containing 10 mM 3-aminotriazole. Under these conditions strong growth is only observed with htra2-beta1, SRp30c, CTD, mCLK2 and SF2/ASF. (**B**) Deletion analysis of rSAF-B-PC. C-terminal deletions were created at the indicated restriction sites. These deletion clones (Δ*Pst*I, Δ*Sma*I, Δ*Stu*I, Δ*Xho*I, Δ*Apa*I) were tested for their ability to interact with full length SRp30c, htra2-beta1, ASF/SF2, mCLK2 and CTD-pol II cDNAs, which were fused to the Gal 4 binding domain. '–' indicates no growth; '+' indicates growth, but <50% of the colonies compared to the full length clone ('++').

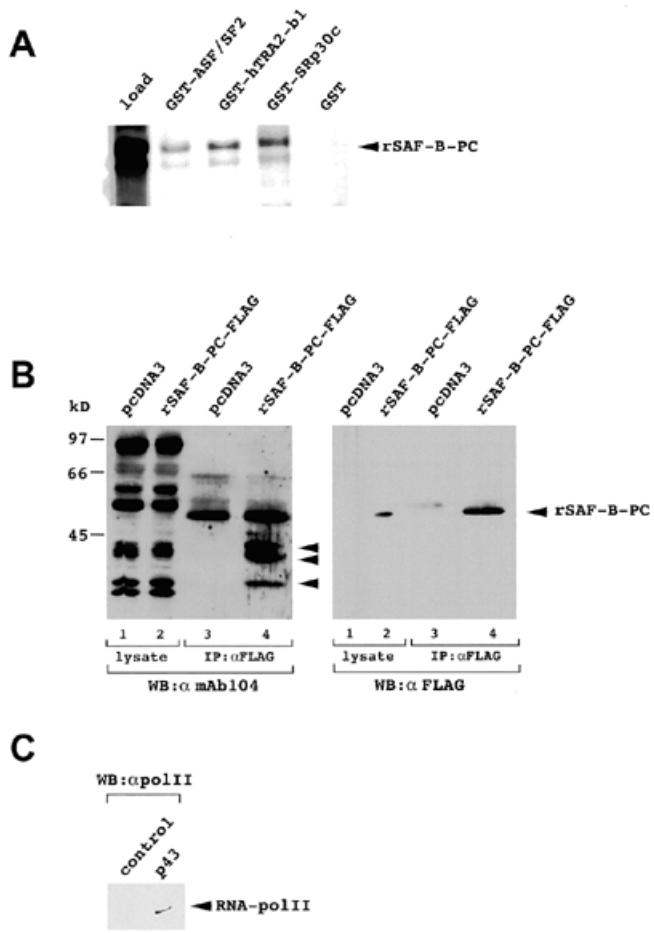

Figure 3. Interaction of rSAF-B-PC with cellular proteins *in vitro*. (**A**) The ^{35}S-labelled protein corresponding the two-hybrid clone (rSAF-B-PC) was transcribed/translated *in vitro* and incubated with GST tagged ASF/SF2, htra2-beta1, SRp30c or pure GST. The proteins retained on glutathione–Sepharose were fractionated on 12% SDS–PAGE gels and autoradiographed overnight. Co-precipitation was observed for all fusion proteins, but not GST alone. (**B**) FLAG-tagged rSAF-B-PC or pcDNA3 (mock) were transiently overexpressed in 293 cells. Cells were lysed and immunoprecipitations were performed with anti-FLAG antibodies. Western blotting was done with mAb104. Left: the blot shows mAb104 antigens between 30 and 45 kDa (arrows), which specifically co-immunoprecipitate with rSAF-B-PC-FLAG. Right: the blot was subsequently stripped and reblotted with anti-FLAG antibodies to confirm the expression of the tagged protein (rSAF-B-PC is indicated by an arrow). (**C**) The human His-tagged fragment p43 was incubated with nuclear extract, washed and separated on SDS–PAGE. The specifically bound RNA polymerase II was detected with an anti-RNA polymerase II antibody.

not GST alone, were shown to interact with rSAF-B-PC (Fig. 3A). Furthermore, after overexpression of FLAG tagged rSAF-B-PC in 293 cells and subsequent immunoprecipitation, we were able to detect co-immunoprecipitating proteins of an apparent molecular weight of 30–45 kDa using the mAb104. As this monoclonal antibody is directed against a common phospho-epitope of several SR proteins (45), these results provide additional evidence for an interaction of SAF-B with SR proteins *in vivo* (Fig. 3B). The interaction of SAF-B with RNA polymerase II was confirmed by a pull down experiment in which a His-tagged human fragment of SAF-B, p43 (Fig. 1A) (38), was incubated with HeLa nuclear extract and Ni–Sepharose in the presence of 30 mM imidazole. Endogenous RNA polymerase II was detected with an anti-RNA-polymerase II antibody in the fraction containing p43, but not in the control fraction (Fig. 3C). Binding of GST tagged ASF/SF2, SRp30c, htra2-beta1 and endogenous RNA polymerase II to rSAF-B-PC (Fig. 3A and C), as well as co-immunoprecipitation of a subset of splicing factors (Fig. 3B), occurred also in the presence of nucleases indicating direct protein–protein interaction (data not shown).

SAF-B forms a ternary complex with SR proteins and S/MAR-DNA *in vitro*

SAF-B was shown to aggregate and precipitate in the presence of S/MAR DNA (38). We employed this feature and precipitated endogenous SAF-B from HeLa nuclear extracts with MII S/MAR DNA and recovered the precipitate by centrifugation. In western blot analysis (Fig. 4) we demonstrate that endogenous SAF-B precipitated together with the same subset of SR proteins we had previously identified in our immunoprecipitation experiments

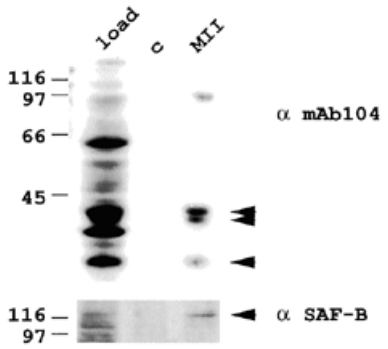

Figure 4. Ternary complex formation of SAF-B, MII S/MAR DNA and splice factors. Nuclear extract was incubated with or without MII S/MAR DNA and centrifuged. The precipitated proteins were then analysed by western blot using SR protein antibody mAb104 (top) and anti-hSAF-B antibody K481 (bottom).

using FLAG-tagged rSAF-B-PC. The identity of SAF-B was further confirmed in a southwestern blot (data not shown). This experiment suggests the existence of a ternary complex containing S/MAR DNA, SAF-B and SR proteins *in vitro*.

Phosphorylation of SAF-B by mCLK2

It is well established that SR proteins interact with other proteins containing an SR-rich region (46,47) and it was proposed that this interaction is regulated by SR protein kinases. hSAF-B is phosphorylated *in vivo* (38) and sequence analysis reveals an enrichment of serine residues in the vicinity of the region mediating the interaction with SR proteins and the CTD-pol II (see above). Several SR protein kinases have been described recently and a family of CDC2-like kinases (CLK) has been implicated in the control of SR protein phosphorylation during interphase (48). A yeast two-hybrid screen using CLK1/STY as a bait was particularly effective to identify several SR proteins or SR-like proteins as substrates. We therefore hoped to find novel splice factors in a yeast two-hybrid screen using CLK2 as a bait. However, in this screen rSAF-B-PC was repeatedly isolated (Fig. 2A) and we mapped the minimal interacting domain to the region encompassing the presumed phosphorylation sites (Fig. 2B). CLK2 and rSAF-B-PC were also shown to co-immunoprecipitate in *in vitro* association experiments (Fig. 5A). Furthermore, when we purified overexpressed mCLK2 from 293 cell lysates using anti-mCLK2 antibodies and used this in an *in vitro* kinase assay with the purified recombinant p43 fragment of hSAF-B as a substrate, we detected phosphorylation by mCLK2, but not by the catalytic mutant mCLK2KR nor mock transfected cells (Fig. 5B). When we determined the identity of the phosphorylated residues by two dimensional phosphoamino acid analysis we found that p43 is phosphorylated by mCLK2 exclusively on serine residues (Fig. 5B). Based on these experiments we conclude that mCLK2 is a potential kinase for SAF-B and can thereby regulate its biological activity. It further suggests that SR proteins are not the exclusive substrates for mCLK2 (34) and opens the possiblity that SAF-B and SR proteins are regulated by the same kinase.

Subnuclear localization of SAF-B and SR proteins *in vivo*

Yeast two-hybrid data and biochemical analysis demonstrated that SAF-B interacts with nuclear proteins. To determine the intracellular localization of SAF-B we tagged our two-hybrid clone with green fluorescent protein and transiently transfected A293 cells with this construct (EGFP-rSAF-B-PC). As shown in Figure 6A, rSAF-B-PC localized throughout the nucleus and created a speckled pattern at points of particular high concentrations. These rSAF-B-PC speckles overlapped with the speckled domains containing SC35, which serves as a general marker to localize SR proteins (Fig. 6B and C). The observed pattern also shows that all the necessary signalling elements to direct SAF-B to the nucleus and speckles is contained within the CTD. Finally, as a fraction of RNA polymerase II was previously shown to be present in nuclear speckles, we conclude that rSAF-B is at least partially localized to the same structures as the proteins it interacts with in genetic and biochemical tests.

SAF-B overexpression can alter splice site selection *in vivo*

It was previously demonstrated that a change in the local concentration of SR proteins can alter the splice site selection *in vivo* (39,49,50) and *in vitro* (40,51). Based on our previous experiments showing an interaction of SAF-B with SR proteins we assumed that highly overexpressed rSAF-B-PC should interfere with the local concentration of SR proteins and therefore change the splice site selection of a splice reporter construct. This could be considered as further evidence for an interaction of SAF-B with SR proteins *in vivo*. Using an E1A minigene construct we observed that the generation of the 10S splice variant increased upon rSAF-B-PC overexpression (Fig. 7). This experiment shows that SAF-B influences the splice site selection *in vivo*, which might be achieved through direct interaction with the spliceosome via SR proteins or by changing the concentration of available SR proteins through sequestration.

SAF-B repressed CAT gene activity

To study the influence of SAF-B on promotor activity and to assess the influence of MAR elements we tested rSAF-B-PC in CAT reporter assays. The CAT reporter was bilaterally flanked with the chicken lysozyme 5′ MAR or by an unrelated sequence of similar base composition (27). At low SAF-B concentrations we observed an inhibitory effect when the reporter was flanked by MAR DNA, but a slight stimulation in the absence of MAR DNA (Fig. 8). This experiment demonstrates that SAF-B influenced transcriptional activity in a MAR-dependent manner, which suggests an *in vivo* association of SAF-B with these elements.

DISCUSSION

We have performed three independent yeast two-hybrid screens, each designed to find novel binding proteins for established molecules such as the splice factor SRp30c, the CTD of the RNA polymerase II and the SR protein kinase CLK2. To our surprise, we found SAF-B as a common interactor. SAF-B was independently isolated as a nuclear scaffold component binding S/MAR DNA (38) and later as a HSP27 promotor binding element in breast cancer cell lines (37). It remains to be seen whether this HSP27 binding site is part of an S/MAR region. A number of recent reports established the existence of macromolecular complexes containing transcriptional and pre-mRNA processing proteins and it was previously shown that splicing complexes are associated to nuclear matrix antigens (52). Based on our two-hybrid screens,

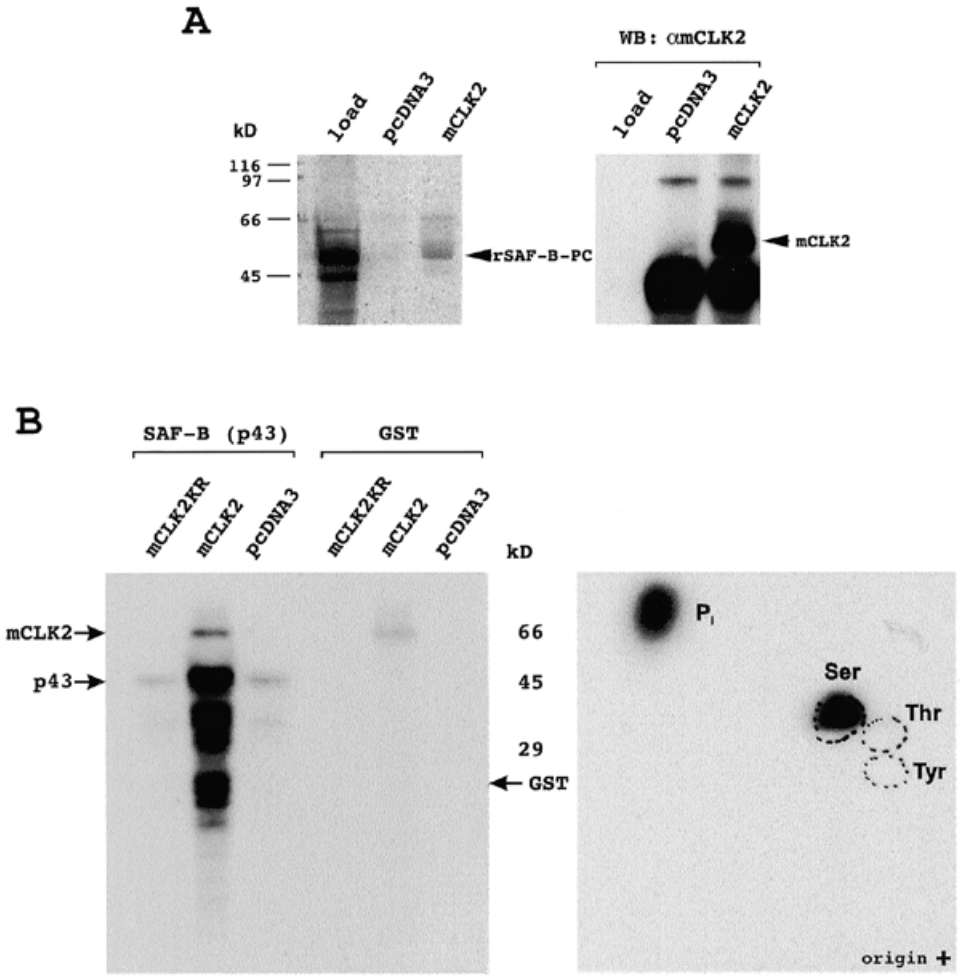

Figure 5. Phosphorylation of SAF-B. (**A**) mCLK2 or pcDNA3 (mock) were transiently overexpressed in 293 cells. *In vitro* transcribed/translated ^{35}S-labelled rSAF-B-PC was added to the lysate. Left: following immunoprecipitation with anti-mCLK2 antibodies, SDS–PAGE and autoradiography, specific co-immunoprecipitation of rSAF-B-PC with mCLK2 was observed. Right: western blot with anti-mCLK2 antibodies shows expression and immunoprecipitation of mCLK2. (**B**) Left: *in vitro* phosphorylation of p43 using mCLK2, mCLK2KR and mock transfected cells as negative control. Right: phosphoamino acid analysis of mCLK2-labelled p43. Only serine residues were labelled.

SAF-B was a potential candidate to represent one of those hitherto uncharacterized antigens.

To further investigate the observed yeast interactions we employed a variety of biochemical and functional assays. One of our main concerns was the possibility of non-specific interactions of the SR domains found in splice factors and the ER-domain of SAF-B. However, C-terminal SAF-B deletion mutants (Fig. 2B) showed that the interaction required additional C-terminal amino acids of SAF-B, making it unlikely that the observed interaction was simply due to an ER–SR association. In contrast, when we deleted the SR domains of the interacting SR protein SF2/ASF, no interaction with SAF-B was detected (Fig. 2B). Furthermore, in overexpression experiments we observed endogenous co-precipitating SR proteins in the molecular weight range expected from the yeast two-hybrid partners, whereas neither SRp55 nor SRp75 were detected. This supports our initial yeast two-hybrid results and further reduces the likelihood that the observed interactions are either RNA-mediated or due to a non-specific SR domain interaction caused by overexpression.

hSAF-B was initially identified through its ability to specifically bind to S/MAR regions and we were able to show that endogenous SAF-B/SR protein complexes can be precipitated with S/MAR DNA (Fig. 4). This suggests the existence of a ternary complex of DNA, SAF-B and SR proteins. Unfortunately, the precipitation of these complexes using DNA and endogenous proteins appeared not efficient enough to detect RNA pol II. However, based on previous association experiments of SR proteins with RNA pol II (16–18) and our results describing an association of SAF-B with RNA pol II, it is conceivable that the polymerase is also part of this complex.

To further elucidate the existence of SAF-B containing protein complexes we used functional assays. Effects of splicing factors on the splicing pattern of an E1A minigene have previously been studied *in vivo*: SF2/ASF promotes the generation of the 13S and hnRNPA1 promotes the usage of the 9S splice form (50). Splice site selection is thought to be governed by an intricate balance of factors (6,47) and the observed change of splice sites mediated by overexpressed SAF-B supports this view. Hence, our experiments

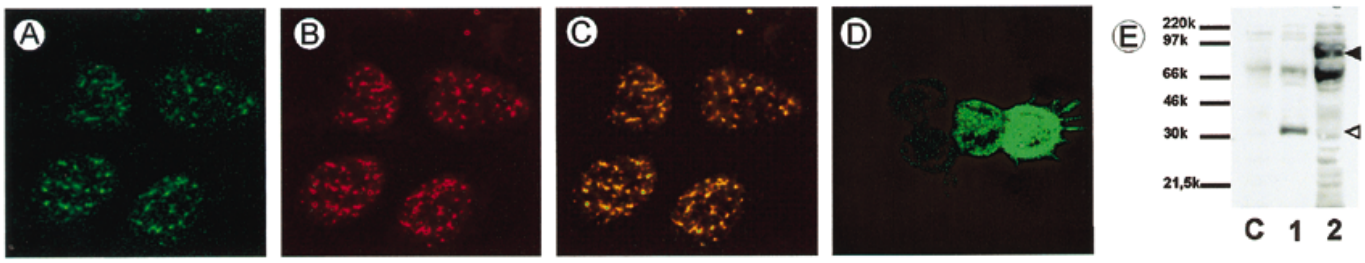

Figure 6. Intracellular distribution of rSAF-B. 293 cells were transiently transfected with EGFP-rSAF-B-PC, fixed and stained with anti-SC35 antibodies. (**A**) The EGFP-rSAF-B-PC distribution appears speckled (green). (**B**) Speckled distribution of SC35 (red). (**C**) The co-localization of EGFP-rSAF-B-PC and SC35 to speckles is seen in the overlay of A and B (orange). (**D**) Control, showing uniform distribution of EGFP throughout the cell. (**E**) Protein expression was determined by western blot using an anti-GFP antibody (Clontech). Both EGFP (open triangle) and EGFP-rSAF-B-PC (closed triangle) gave rise to protein bands of the expected size (29.3 and 75 kDa, C mock transfected A293 cells).

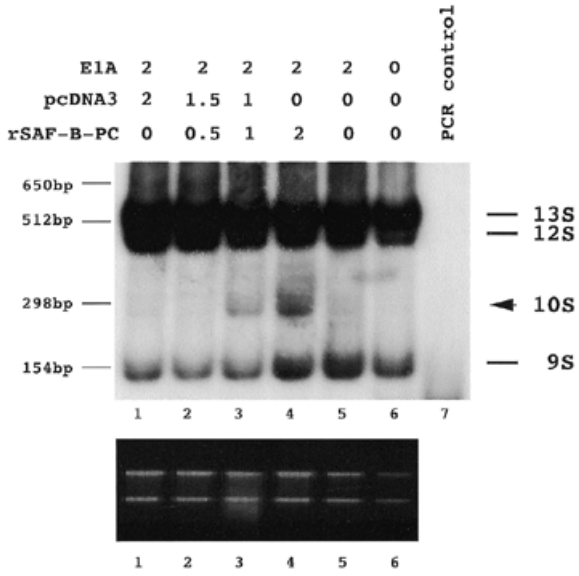

Figure 7. Influence of SAF-B on E1A alternative splicing. Top: representative figure of alternative splicing of an E1A minigene with increasing amounts (μg) of rSAF-B-PC (as indicated on the top). pcDNA3 was included to ensure that the same amount of DNA was added in each experiment. The increased 10S splice product is indicated by an arrow. Other splice products are marked with lines. In the PCR control, no reverse transcribed RNA was added. The identity of the 10S splice variant was confirmed by Southern blotting using 10S-specific primers (not shown). Bottom: ethidium bromide stained gel of RNA, indicating that equal amounts of RNA were used for the RT–PCR.

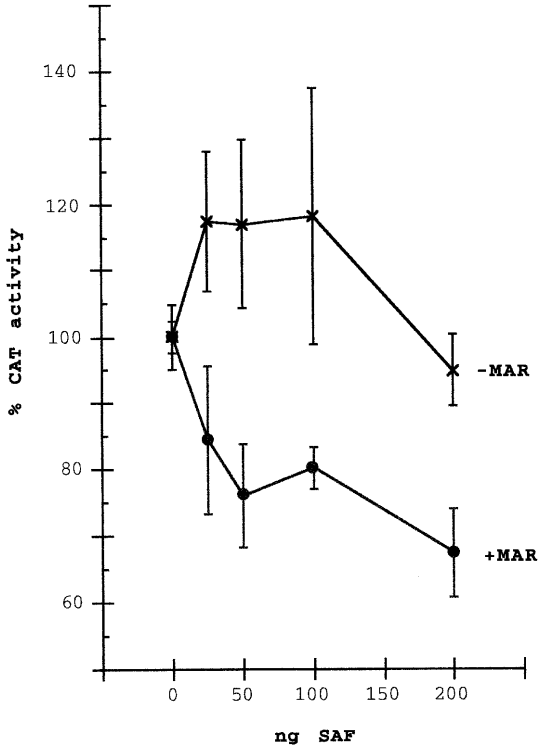

Figure 8. Influence of SAF-B on transcription. NIH 3T3 cells were transiently transfected with increasing amounts of SAF-B and a fixed amount of the reporter MAR-lysozyme promotor-CAT-MAR construct or a control reporter construct containing non-MAR sequences. Data represents means ± SD of four experiments.

provide further evidence for an *in vivo* association of SAF-B with splice factors.

We also used CAT reporter constructs and found a repression of gene activity in an S/MAR-dependent manner. This effect is reminiscent of the transcriptional repression activity of other S/MAR binding proteins such as MeCP2/ARBP and SATB1 (30,53–56). Furthermore, SAF-B-mediated repression of gene activity was also detected in a previous report using a HSP27 promotor element (37). It is well documented that MARs insulate and enhance gene expression in stable cellular expression systems and in transgenic animals and plants (27–29,57,58). However, in transient expression systems, they have been reported to behave as neutral elements or to repress transcription (57,59).

Our data suggest that overexpressed SAF-B might repress transcription by tethering the transcription complex to S/MAR elements. To summarize, the effects of SAF-B on splicing and transcription in transient transfection assays suggest that the observed *in vitro* binding properties are relevant *in vivo*.

Co-localization of SAF-B and SR proteins further underlines the observed molecular interactions, as recent reports demonstrated that several splicing components are located in speckles from which they are released upon demand (15). It is therefore possible that SAF-B is also stored in speckles and that the functional association of SAF-B and SR proteins may not necessarily reside

there but rather at sites of active transcription. The co-localization might also explain that both molecules are substrates for the SR protein kinase CLK2, which is supposed to release speckle components into the nucleoplasm through phosphorylation (34).

Recent work has demonstrated an association of RNA polymerase II with capping components, the 3′-processing machinery and splicing factors (16–18). This supports the idea of an RNA 'factory' in which splicing, transcription and polyadenylation are coupled. Our binding data and the demonstration of a ternary complex of SAF-B, splicing components and S/MAR DNA (Fig. 4) as well as binding of rSAF-B-PC to RNA polymerase II (Fig. 3C) adds to this model a molecule with the potential to target an 'RNA factory' to S/MAR elements near transcriptionally active regions, thereby forming a 'transcriptosome' (60).

In this model, the most likely role of SAF-B is structural, despite some evidence that SAF-B is involved in transcriptional regulation *in vivo* (Fig. 8) (37). However, as both studies use overexpression experiments this possibility requires more detailed investigations, although S/MAR elements were previously shown to play a role in tissue-specific gene expression (29) and the T-cell-specific S/MAR binding protein SATB1 was shown to be crucial for T-cell development (30). Furthermore, there are several examples where changes in chromatin architecture (24) influenced gene expression in a tissue-specific way. It is thus tempting to speculate that there may be a general role for S/MAR binding proteins in the control of constitutive, tissue-specific or developmentally controlled gene expression.

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REFERENCES

1 Ptashne,M. and Gann,A. (1997) *Nature*, **386**, 569–577.
2 Narayan,P. and Rottman,F.M. (1992) *Adv. Enzymol.*, **65**, 255–285.
3 Gorlich,D. and Mattaj,I.W. (1996) *Science*, **271**, 1513–1518.
4 Sachs,A.B. (1993) *Cell*, **74**, 413–421.
5 Ruiz-Echevarria,M.J., Czaplinski,K. and Peltz,S. (1996) *Trends Biol. Sci.*, **21**, 433–437.
6 Krämer,A. (1996) *Annu. Rev. Biochem.*, **65**, 367–409.
7 Will,C.L. and Lührmann,R. (1997) *Curr. Opin. Cell Biol.*, **9**, 320–328.
8 Raska,I. (1995) *J. Cell Biochem.*, **59**, 11–26.
9 Nickerson,J.A., Blencowe,B.J. and Penman,S.B. (1995) *Int. Rev. Cyt.*, **162A**, 67–123.
10 van Driel,R., Wansink,D.G., van Steensel,B., Grande,M.A., Schul,W. and de Jong,L. (1995) *Int. Rev. Cyt.*, **162A**, 151–189.
11 Beyer,A.L. and Osheim,Y.M. (1988) *Genes Dev.*, **2**, 754–765.
12 Xing,Y., Johnson,C.V., Moen,P.T., McNeil,J.A. and Lawrence,J.B. (1995) *J. Cell. Biol.*, **131**, 1635–1647.
13 Huang,S. and Spector,D.L. (1996) *J. Cell. Biol.*, **133**, 719–732.
14 Spector,D.L. (1993) *Annu. Rev. Cell. Biol.*, **9**, 265–315.
15 Misteli,T., Cáceres,J.F. and Spector,D.L. (1997) *Nature*, **387**, 523–527.
16 McCracken,S., Fong,N., Yankulov,K., Ballantyne,S., Pan,G., Greenblatt,J., Patterson,S.D., Wickens,M. and Bentley,D.L. (1997) *Nature*, **385**, 357–361.
17 Du,L. and Warren,L. (1996) *J. Cell. Biol.*, **136**, 5–18.
18 Kim,E., Du,L., Bregman,D.B. and Warren,S.L. (1996) *J. Cell. Biol.*, **136**, 19–28.
19 Neugebauer,K.M. and Roth,M.B. (1997) *Genes Dev.*, **11**, 3279–3285.
20 Wolffe,A.P. (1992) *Chromatin: Structure and Function.* Academic Press, London.
21 Jeppesen,P. (1997) *Bioessays*, **19**, 67–74.
22 Siegfried,Z. and Cedar,H. (1997) *Curr. Biol.*, **7**, R305–R307.
23 Tate,P.H. and Bird,A.P. (1993) *Curr. Opin. Genet.*, **3**, 226–231.
24 Cairns,B.R. (1998) *Trends Biochem. Sci.*, **23**, 20–25.
25 Gasser,S.M. and Laemmli,U.K. (1987) *Trends Genet.*, **3**, 16–22.
26 Bode,J., Kohwi,Y., Dickinson,L., Joh,T., Klehr,D., Mielke,C. and Kohwi-Shigematsu,T. (1992) *Science*, **255**, 195–197.
27 Phi-Van,L. and Strätling,W.H. (1996) *Biochemistry*, **35**, 10735–10742.
28 Boulikas,T. (1995) *Int. Rev. Cyt.*, **162A**, 279–388.
29 Forrester,W.C., van Gerderen,C., Jenuwein,T. and Grosschedl,R. (1994) *Science*, **265**, 1221–1225.
30 Dickinson,L.A., Joh,T., Kohwi,Y. and Kohwi-Shigematsu,T. (1992) *Cell*, **70**, 631–645.
31 Beil,B., Screaton,G. and Stamm,S. (1997) *DNA Cell Biol.*, **16**, 679–690.
32 Geertman,R., McMahon,A. and Sabban,E.L. (1996) *Biochim. Biophys. Acta*, **1306**, 147–152.
33 Bourquin,J.-P., Stagljar,I., Meier,P., Moosmann,P., Silke,J., Baechi,T., Georgiev,O. and Schaffner,W. (1997) *Nucleic Acids Res.*, **25**, 2055–2061.
34 Nayler,O., Stamm,S. and Ullrich,A. (1997) *Biochem. J.*, **326**, 693–700.
35 Geer,P.v.d. and Hunter,T. (1994) *Electrophoresis*, **15**, 554–558.
36 Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
37 Oesterreich,S., Lee,A.V., Sullivan,T.M., Samuel,S.K., Davie,J.R. and Fuqua,S.A.W. (1997) *J. Cell. Biochem.*, **67**, 275–286.
38 Renz,A. and Fackelmayer,F.O. (1996) *Nucleic Acids Res.*, **24**, 843–849.
39 Screaton,G.R., Caceres,J.F., Mayeda,A., Bell,M.V., Plebanski,M., Jackson,D.G., Bell,J.I. and Krainer,A.R. (1995) *EMBO J.*, **14**, 4336–4349.
40 Mayeda,A. and Krainer,A.R. (1992) *Cell*, **68**, 365–375.
41 Ge,H., Zuo,P. and Manley,J.L. (1991) *Cell*, **66**, 373–382.
42 Zamore,P.D., Patton,J.G. and Green,M.R. (1992) *Nature*, **355**, 609–614.
43 Fu,X.D. and Maniatis,T. (1992) *Science*, **256**, 535–538.
44 Schmauss,C., McAllister,G., Ohosone,Y., Hardin,J.A. and Lerner,M.R. (1989) *Nucleic Acids Res.*, **17**, 1733–1743.
45 Roth,M.B., Murphy,C. and Gall,J.G. (1990) *J. Cell. Biol.*, **111**, 2217–2223.
46 Fu,X.-D. (1995) *RNA*, **1**, 663–680.
47 Manley,J.L. and Tacke,R. (1996) *Genes Dev.*, **10**, 1569–1579.
48 Colwill,K., Pawson,T., Andrews,B., Prasad,J., Manley,J.L., Bell,J.C. and Duncan,P.I. (1996) *EMBO J.*, **15**, 265–275.
49 Wang,J. and Manley,J.L. (1995) *RNA*, **1**, 335–346.
50 Cáceres,J., Stamm,S., Helfman,D.M. and Krainer,A.R. (1994) *Science*, **265**, 1706–1709.
51 Ge,H. and Manley,J.L. (1990) *Cell*, **62**, 25–34.
52 Blencowe,B.J., Nickerson,J.A., Issner,R., Penman,S. and Sharp,P.A. (1994) *J. Cell. Biol.*, **127**, 593–607.
53 Nan,X., Campoy,F.J. and Bird,A. (1987) *Cell*, **88**, 471–481.
54 Weitzel,J.M., Buhrmester,H. and Strätling,W.H. (1997) *Mol. Cell. Biol.*, **17**, 5656–5666.
55 Liu,J., Bramblett,D., Zhu,C., Lozano,M., Kobayashi,R., Ross,S.R. and Dudley,J.P. (1997) *Mol. Cell. Biol.*, **17**, 5275–5287.
56 Kohwi-Shigematsu,T., Maass,K. and Bode,J. (1997) *Biochemistry*, **36**, 12005–12010.
57 Stief,A., Winter,D., Strätling,W.H. and Sippel,A. (1989) *Nature*, **341**, 343–345.
58 Namciu,S.J., Blochinger,K.B. and Fournier,R.E.K. (1988) *Mol. Cell. Biol.*, **18**, 2382–2391.
59 Hörtnagel,K., Mauter,J., Strobl,L.J., Wolf,D.A., Christoph,B., Geltinger,C. and Polack,A. (1995) *Oncogene*, **10**, 1393–1401.
60 Corden,J.L. and Patturajan,M. (1997) *Trends Biochem. Sci.*, **22**, 413–416.