FRET analysis of splicing factors involved in exon and intron definition in living cells

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PhD

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2007

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Declaration

I declare this thesis was composed by myself. The contributions of others to the work are clearly indicated. This work has not been submitted for any other degree or professional qualification.

Jonathan Ellis

September 2007

Acknowledgements

I am grateful to Javier Caceres, my supervisor, for guidance and support throughout my PhD. I would also like to thank him for comments and constructive criticism during the preparation of this thesis. I would also like to thank all the other members of Caceres lab, past and present that I have worked with during the course of this Ph.D. They have all been very helpful, providing guidance and support and making the lab a very enjoyable place to work. In particular, I would like to thank Jeremy Sanford for his assistance in the lab during the first two years of my PhD.

The work in this thesis was carried out as part of a collaboration with Angus Lamond's lab at the university of Dundee. I am very grateful to David Lleres, his comments and technical assistance were of invaluable help to complete this PhD. Sam Swift has also been very helpful in providing technical support with the microscopes.

I would also like to thank Nick Gilbert for his help and useful comments and Paul Perry for his assistance with the microscopes in Edinburgh. John Ireland has been very helpful in assisting me with a bioinformatic analysis of the data. Finally I would like to thank my mum and dad for their support and encouragement in everything I do.

Abstract

I have analyzed the interactions between SR proteins and splicing components that are bound at the 5' or 3' splice site using fluorescence resonance energy transfer (FRET) microscopy. The SR proteins interact with the U1 snRNP-associated 70 kDa protein (U170K) at the 5'splice site and with the small subunit of the U2 snRNP auxiliary factor (U2AF35) at the 3' splice site. These interactions have been extensively characterized biochemically in the past, and are proposed to play roles in both intron and exon definition. We employed FRET acceptor photobleaching and fluorescence lifetime imaging microscopy (FLIM) to identify and spatially localise sites of direct interactions of SF2/ASF, and other SR proteins, with U2AF35 and U1-70K in live cell nuclei. These interactions were shown to occur more strongly in interchromatin granule clusters (IGCs). They also occur in the presence of the RNA polymerase II inhibitor, DRB, demonstrating that they are not exclusively co-transcriptional. FLIM data have also revealed a novel interaction between HCC1, a factor highly related to the large subunit of the U2AF splicing factor, with both subunits of U2AF that occur in discrete domains within the nucleoplasm but not within IGCs. These data demonstrate that the interactions defining intron and exon definition do occur in living cells in a transcription-independent manner**.**

Abbreviations

A	Adenine
AMCA	7-amino-4-methylcoumarin-3-acetic acid
ATP	Adenosine triphosphate
^{o}C	Centigrade
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CFP	Cyan fluorescent protein
CMV	Cyto megalo virus
C-terminal	Carboxy-terminal
Ca^{2+}	Calcium ions
cGMP	Cyclic guanosine monophosphate
Da	Daltons
Δ	Delta
DNA	Deoxyribonucleic acid
DRB	5,6-dichloro-1-b-d-ribofuranosylbenzimidazole
EDTA	Ethylenediaminotetra-acetic acid
ECFP	Enhanced cyan fluorescent protein
EGFP	Enhanced green fluorescent protein
EYFP	Enhanced yellow fluorescent protein
g	Grammes
G	Guanine
GFP	Green fluorescent protein
hnRNP	Heterogenous ribonucleoprotein particle
HRP	Horse raddish peroxidase
hz	Hertz
h	Hour
IgG	Immunoglobulin G
K	Kilo
KCl	Pottasium chloride
l	Litre
m	Mili
μ	Micro
M	Molar
mAb	Monoclonal antibody

min	Minutes
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein particle
n	nano
ns	nanosecond
NaCl	Sodium Chloride
N-terminal	Amino-terminal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
R	Purine
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RT	Room temperature
SDS	Sodium dodecyl sulphate
Sec	Seconds
snRNA	Small nuclear ribonucleic acid
snRNP	Small nuclear ribonucleoprotein particle
Spec	Spectrometry
TxRed	Texas Red
Y	Pyrimide
YFP	Yellow fluorescent protein

Introduction

1.1 Gene Expression

The expression of eukaryotic protein-encoding genes begins in the nucleus with transcription by RNA polymerase II (pol II) to make mRNA precursors (pre-mRNAs). Rather than a simple linear pathway of gene expression, numerous studies have demonstrated extensive coupling between the stages of gene expression (reviewed by (Maniatis and Reed, 2002)). During transcription, the nascent pre-mRNA is capped at the 5' end, noncoding intervening sequences called introns are removed by splicing and the 3' end is cleaved and polyadenylated. The mature mRNA is then released from the site of transcription and exported to the cytoplasm for translation. In addition to this pathway, an RNA surveillance system eliminates aberrantly processed or mutant pre-mRNAs and mRNAs. Distinct multi-component cellular complexes carry out each stage of the gene expression pathway. These multi-component cellular complexes interact physically and functionally with one another. In the nucleus the carboxy-terminal domain (CTD) of pol II coordinates many RNA processing events by providing a platform for factors involved in different steps of RNA processing. The importance of the CTD is illustrated by the effect of certain CTD deletions that do not inactivate transcription but significantly decrease the efficiency of capping, splicing and polyadenylation (Cho et al., 1997; McCracken et al., 1997a; McCracken et al., 1997b). In addition pol II CTD stimulates splicing in human cells independently of its effects on capping or 3' end formation (Fong and Bentley, 2001).

1.2 Pre-mRNA splicing

A ubiquitous feature of eukaryotes is the presence of intervening sequences that interrupt coding regions of genes. Nuclear pre-mRNA splicing is the process by which these noncoding intervening sequences (introns) in messenger RNAs are precisely removed and the functional coding sequences (exons) ligated to generate mature, translatable mRNAs. The complexity of the process and the number of spliced genes increases with the complexity of the organism. *Saccharomyces cerevisiae* have introns in only about 250 of more than 5000 genes, and they are largely devoid of alternative splicing. However, more than 25% of mRNAs are spliced because many highly expressed genes, mainly encoding ribosomal proteins, contain introns (Ares, Jr. et al., 1999). The regulation of splicing is important to several aspects of yeast biology including meiosis, ribosome biogenesis and mRNA export (Dabeva et al., 1986; Nandabalan and Roeder, 1995; Rodriguez-Navarro et al., 2002; Preker and Guthrie, 2006). Components of the yeast spliceosome have also been shown to play an important role in regulating cell cycle progression (Ben Yehuda et al., 2000; Russell et al., 2000). In humans more than 99% of genes contain introns.

Splicing proceeds via two transesterification reactions (Figure 1.1). In the first reaction, the 2' hydroxyl group of an intron adenosine residue attacks the 5' splice site phosphodiester bond, producing a branched lariat intermediate structure and a free 5' exon. In the second reaction, the 3' hydroxyl group of the 5' exon attacks the 3' splice site phosphodiester bond, producing ligated exons and an excised intron lariat.

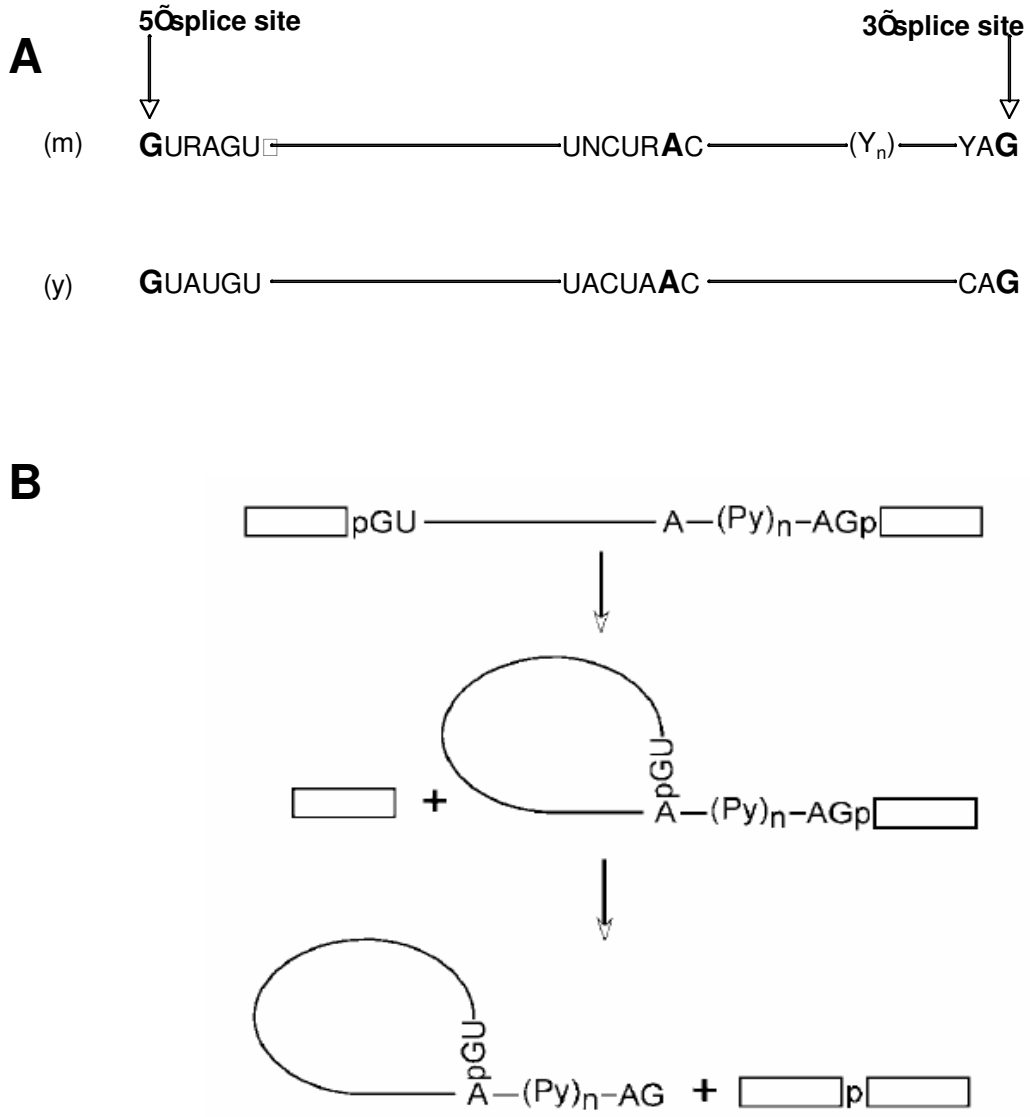


Figure 1.1: (A) Conserved sequences in nuclear pre-mRNA introns. The 5' and 3' splice site and the branch-site consensus sequences are shown (R: purines; Y: pyrimidines). The extremely conserved terminal nucleotides and the branch nucleotide of introns are indicated by enlarged letters. Mammalian introns (m) usually contain a stretch of pyrimidine residues, called the polypyrimidine tract, near the 3' splice site, whereas not all yeast (y) introns have such a sequence (Tarn and Steitz, 1997). **(B) Two-step chemical mechanism for pre-mRNA splicing.** Splicing takes place in two transesterification steps. The first step results in two reaction intermediates: the detached 5' exon and an intron/3' exon fragment in a lariat structure. The second step ligates the two exons and releases the lariat intron (Black, 2000).

1.3 Alternative splicing

In violation of the 'one gene, one polypeptide' rule, alternative splicing allows individual genes to produce multiple protein isoforms – thereby playing a central part in generating proteomic diversity (reviewed by (Black, 2000; Matlin et al., 2005)). The *Drosophila* Dscam gene exemplifies the extreme structural diversity that is achievable by alternative splicing. Dscam is a cell surface protein that is involved in axon guidance in the developing brain, and can potentially generate up to 38,016 alternatively spliced isoforms (Celotto and Graveley, 2001; Schmucker and Flanagan, 2004).

Alternative splicing has assumed a high profile recently owing to the dual realisation that there are fewer human genes than originally anticipated, and that alternative splicing is more the rule than the exception. Analyses of expressed sequence tag (EST) and cDNA datasets conservatively estimated that about 40–60% of human genes are alternatively spliced and this number increased to 73% when alternative splicing microarray data was combined with ESTs (Johnson et al., 2003).

The mechanisms of splice-site selection in alternative and constitutive splicing appear to be closely related because components of the splicing apparatus essential for the constitutive splicing reaction, also have a role in the regulation of alternative splicing (reviewed by (Horowitz and Krainer, 1994)). Alternative exons often have suboptimal splice sites and/or a suboptimal length when compared to constitutive exons. Splicing of regulated exons is modulated by trans-acting factors that recognise an arrangement of positive (splicing enhancers) and/or negative (splicing silencers) cis-acting RNA elements, which can be either exonic or intronic. These auxiliary elements are involved in defining both constitutive and alternative exons.

The importance of accurate splicing is illustrated by the fact that at least 15% (Krawczak et al., 1992), and perhaps as many as 50% (Pagani and Baralle, 2004), of human genetic diseases arise from mutations either in consensus splice site sequences or in the more variable auxiliary elements known as exon and intron splicing enhancers (ESEs and ISEs) and silencers (ESSs and ISSs) (Blencowe, 2000; Philips and Cooper, 2000; Caceres and Kornblihtt, 2002).

1.4 Co-transcriptional splicing

Pre-mRNA splicing often occurs co-transcriptionally (reviewed by (Bentley, 2002; Neugebauer, 2002; Kornblihtt et al., 2004)) but may be completed post-transcriptionally, as demonstrated by electron microscopy studies of the Balbiani ring genes of *Chironomus tentans*, in which a high proportion of nascent RNAs lack introns at their 5' ends but still contain terminal introns (Bauren and Wieslander, 1994; Wetterberg et al., 1996). Co-transcriptional splicing has also been documented in *Drosophila* (Osheim et al., 1985; Beyer and Osheim, 1988; LeMaire and Thummel, 1990) and humans (Wuarin and Schibler, 1994; Tennyson et al., 1995). In yeast pre-mRNA splicing is predominantly post-transcriptional (Tardiff et al., 2006). Recent evidence demonstrates that transcription promotes splicing and, reciprocally, that splicing promotes transcription (Ghosh and Garcia-Blanco, 2000; Fong and Bentley, 2001; Kwek et al., 2002; Hicks et al., 2006; Das et al., 2006; 2007). Spliceosomal U small ribonucleoproteins (U snRNPs) form a complex with the elongation factor TAT-SF1 and this complex stimulates both *in vitro* transcriptional elongation and splicing (Fong and Zhou, 2001). Splicing can occur post-transcriptionally *in vivo* and

in vitro, but linking splicing to transcription is thought to maximize its fidelity and efficiency (Howe et al., 2003).

Numerous studies have been carried out to identify splicing factors that interact with pol II, the CTD, or other components of the transcription machinery. Splicing factors or splicing related proteins reported to interact with pol II include SR-related-CTD-associated factors (SCAFs), PSF/p54nrb, U2 snRNP auxiliary factor (U2AF), members of the serine- arginine-rich proteins (SR proteins), and one or more of the five spliceosomal U snRNPs (Mortillaro et al., 1996; Yuryev et al., 1996; Corden and Patturajan, 1997; Kim et al., 1997; Patturajan et al., 1998; Morris and Greenleaf, 2000; Robert et al., 2002; Emili et al., 2002; Kameoka et al., 2004; Ujvari and Luse, 2004). A proteomic analysis of factors associated with immunopurified human pol II identified the SR protein family of splicing factors and all of the components of U1 snRNP, but no other snRNPs or splicing factors (Das et al., 2007). The association of SR proteins with pol II positions these splicing factors close to the nascent pre-mRNA. Thus, these factors out-compete inhibitory hnRNP proteins, resulting in efficient spliceosome assembly on nascent pol II transcripts. The cotranscriptional recruitment of SR proteins and U1 snRNP would be expected to increase the fidelity of the earliest recognition of the 5' splice site in nascent pre-mRNAs.

1.5 Spliceosome assembly

Pre-mRNA splicing occurs in the spliceosome, a large ribonucleoprotein complex consisting of five small ribonucleoproteins (U1, U2, U4/U6 and U5 snRNPs) and numerous non-snRNP splicing factors (reviewed by (Kramer, 1996)).

The spliceosome assembles de novo on the pre-mRNA in a coordinated series of intricate movements by binding sequences located at the 5' and 3' ends of introns (Figure 1.1). For most introns the 5' and 3' splice sites are defined by the consensus AG/GURAGU (R = purine) and Y/AG (where / denotes the exon/intron boundary, Y = pyrimidine) respectively (reviewed by (Horowitz and Krainer, 1994)). The presence of a 10- to 20-nucleotide polypyrimidine tract between the branch point (consensus sequence YNYURAC) and the AG dinucleotide aids the recognition of the 3' splice site. In contrast to the degenerate nature of mammalian splice site signals yeast have highly conserved splice sites and branchpoints. Splice sites are recognised as pairs either across exons or introns, depending on which distance is shorter. For example, yeast genes have very small introns and recognition of exons seems to occur by interactions mediated across the intron itself, in a process known as intron definition (Abovich and Rosbash, 1997; Romfo et al., 2000; Lim and Burge, 2001). Intron definition is also the predominant mechanism in splicing of small *Drosophila* introns (Talerico and Berget, 1994). In contrast, the correct identification of exons is a complex problem in vertebrate genes, which have small exons separated by large introns (Black, 1995). In this case, exon definition is facilitated by interactions between the upstream 3' splice site and the downstream 5' splice site (Robberson et al., 1990; Berget, 1995).

Pre-spliceosome assembly is initiated by the stable associations of (i) U1 snRNP with the 5' splice site (ii) branchpoint-binding protein SF1 with the branchpoint, and (iii) U2 snRNP auxiliary factor (U2AF) with the polypyrimidine tract (E complex) (reviewed by (Reed, 2000)) (Figure 1.2). Binding of SF1 to the branchpoint is weak but the affinity is increased by a simultaneous interaction with U2AF65 (Berglund et al., 1998). These early events are ATP-independent and commit the pre-mRNA to the splicing pathway. ATP hydrolysis then leads to the formation of the A complex, which is characterised by the stable association of U2 snRNP at the branchpoint. U2-snRNP binding to the branch point requires auxiliary factors SF1 and U2AF (Guth and Valcarcel, 2000). U2AF is a heterodimer of 35 and 65-kDa subunits (Ruskin et al., 1988; Zamore and Green, 1989). U2AF65 binds to the polypyrimidine tract through its RNA recognition motifs (RRMs) and contacts the branch point via its RS domain (Zamore et al., 1992; Gaur et al., 1995; Valcarcel et al., 1996) whereas U2AF35 binds to the AG dinucleotide at the 3' splice site (Zhang et al., 1992; Merendino et al., 1999b; Wu et al., 1999; Zorio and Blumenthal, 1999). The interaction between U2AF35 and the AG dinucleotide can stabilise the binding of U2AF65 to weak polypyrimidine tract characteristic of AG-dependent pre-mRNAs (Guth et al., 1999a). U2AF35 also mediates arginine-serine (RS) domain dependent bridging interactions with SR proteins (Wu and Maniatis, 1993).

The B complex is formed by the incorporation of the U4/U6•U5 tri-snRNP. Subsequently, dramatic structural rearrangements within the spliceosome driven by several ATP-dependent RNA helicases occur. The duplex between U4 and U6 snRNAs is unwound, and U1 snRNA base pairing at the 5' splice site is replaced by base pairing of the U6 snRNA. These rearrangements are thought to be critical for formation of the catalytically active spliceosomal C complex. These spatial

rearrangements are necessary to reposition the splicing intermediates generated in the first catalytic step so that the reactive groups involved in the second catalytic step are brought closer together (reviewed by (Will and Luhrmann, 2001; Nilsen, 2003). Following the second catalytic step ATP-dependent RNA helicases mediate the release of the spliced mRNA from the spliceosome resulting in spliceosome disassembly (Arenas and Abelson, 1997; Schwer and Gross, 1998).

Like many mRNA processing factors, the spliceosome is recruited during transcription. Chromatin immunopreciptation (ChIP) experiments in both mammalian and yeast systems have shown that spliceosome components assemble *in vivo* on intron- containing genes in a stepwise manner consistent with *in vitro* studies of splicing complexes (Kotovic et al., 2003; Gornemann et al., 2005; Lacadie and Rosbash, 2005; Lacadie et al., 2006; Listerman et al., 2006; Tardiff and Rosbash, 2006). The elongation rate of pol II has been shown to influence cotranscriptional splicosome assembly (Listerman et al., 2006). In yeast spliceosomal assembly completes posttranscriptionally in most cases (Moore et al., 2006).

This model of spliceosome assembly in which spliceosomal snRNAs associate with the pre-mRNA in a stepwise, ordered manner is challenged by the holospliceosome model, in which all spliceosomal snRNPs preassemble into a penta-snRNP complex (Stevens et al., 2002). Evidence for this model comes from observations of an early interaction between the U4/U6•U5 tri-snRNP and the 5' splice site that occurs prior to stable binding of the U2-snRNP to the pre-mRNA branch point (Konforti et al., 1993; Konforti and Konarska, 1994; 1995; Maroney et al., 2000; Johnson and Abelson, 2001). Several observations suggest that the U1 and/or U2snRNPs interact with the U4/U6•U5 tri-snRNP prior to engaging the pre-mRNA (Konarska and Sharp, 1988; Hausner et al., 1990; Wassarman and Steitz,

1992). More recent studies revealed that a large 200S complex containing the U1, U2, U4, U5, and U6 snRNPs can assembly onto a short RNA containing a 5' splice site in HeLa cell nuclear extracts, consistent with the existence of a human penta-snRNP (Malca et al., 2003). In *Saccharomyces cerevisiae* a pre-formed penta-snRNP was shown to function as an intact entity in *in vitro* splicing assays when supplemented with micrococcal nuclease-treated extract (Stevens et al., 2002). Based on this evidence it is proposed that the holospliceosome engages the pre-mRNA. In subsequent remodelling steps, initial weak contacts between the spliceosome and the pre-mRNA are progressively stabilised and a catalytically active spliceosome is formed. It is proposed that due to the relatively harsh conditions used to investigate spliceosome assembly *in vitro* the various assembly intermediates that are observed simply reflect the stepwise stabilisation of snRNP-premRNA interactions, rather than a stepwise recruitment of snRNPs. Thus according to the holospliceosome model, spliceosomal complexes such as the A complex arise by disassociaton of the holospliceosome. However it has been demonstrated that holosplicesome formation is not a prerequisite for generating catalytically active human spliceosomes and that, at least *in vitro*, the U1 and U2 snRNPs can functionally associate with the pre-mRNA, prior to and independent of the tri-snRNP (Behzadnia et al., 2006).

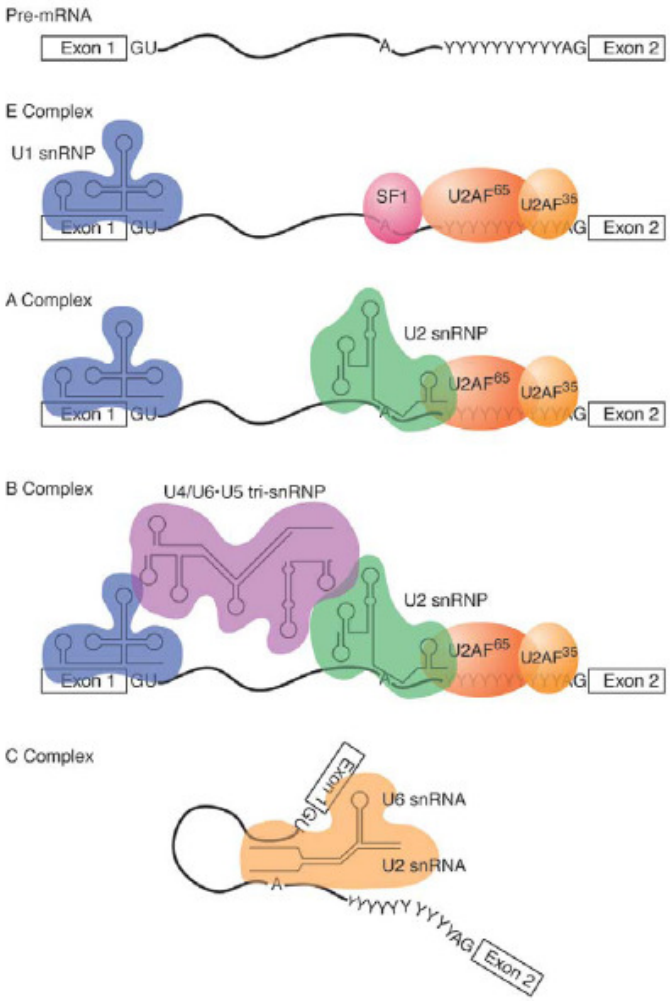


Figure 1.2: Spliceosome assembly pathway. See text for description (Hertel and Graveley, 2005)

1.6 Spliceosome composition

Biochemical purifications from yeast and HeLa nuclear extracts have been instrumental in defining the protein composition of the spliceosome (reviewed by (Jurica and Moore, 2003)). Much has been learned about the protein components of the spliceosome from analysis of individual purified small nuclear ribonucleoproteins (reviewed by (Will and Luhrmann, 2001)) and salt-stable spliceosome particles (Bennett et al., 1992; Neubauer et al., 1998). The advent of pre-mRNAs containing aptamer sequences (e.g., tobramycin) or binding sites for an affinity-tagged RNA binding protein (e.g., viral MS2-MBP) has made it possible to elute splicing complexes under nondenaturing conditions (Wang and Rando, 1995; Das et al., 2000). This has lead to the purification and mass spec analysis of individual subcomplexes (Hartmuth et al., 2002; Jurica et al., 2002; Makarov et al., 2002). A proteomic analysis of the human spliceosome identified about 145 distinct spliceosomal proteins, making the spliceosome the most complex cellular machine so far characterised (Zhou et al., 2002a). While the U snRNPs are at the heart of this complex machinery, a myriad of non-snRNP-associated splicing factors, such as SR proteins and DexD/H box ATPases interact sequentially with the pre-mRNA. The human spliceosome contains at least 30 proteins with putative or known roles in steps of gene expression other than splicing (Rappsilber et al., 2002; Zhou et al., 2002a). These factors may be required for mediating the extensive coupling between splicing and other steps in gene expression.

The size and three-dimensional structure of the intermediate stages of spliceosome assembly and the small nuclear ribonucleoproteins have been visualised by cryo-electron microscopy (Reed et al., 1988; Stark et al., 2001; Zhou et al., 2002b;

Azubel et al., 2004; Boehringer et al., 2004; Jurica et al., 2004; Deckert et al., 2006; Sander et al., 2006; Cohen-Krausz et al., 2007).

1.7 SR proteins

The SR proteins are a highly conserved family of structurally and functionally related non-snRNP splicing factors present in all metazoans (reviewed by (Fu, 1995; Graveley, 2000; Sanford et al., 2003)). Biochemical experiments have provided strong evidence that SR proteins play essential roles in general, or constitutive splicing. But they seem to be equally important in splicing regulation, being able to modulate selection of alternative splice sites in a concentration-dependent manner and to contribute to activation (or repression) of splicing through interactions with exon and intron splicing enhancers (ESEs and ISEs) (or silencers (ESSs or ISSs) (Lavigueur et al., 1993; Sun et al., 1993; Tian and Maniatis, 1993). Given their crucial role in constitutive splicing, it is somewhat surprising that SR proteins appear not to be conserved in *Saccharomyces cerevisiae* although they are present in *Schizosaccharomyces pombe* (reviewed by (Kaufer and Potashkin, 2000)). The fact that branchpoints and 5' splice sites strongly match the consensus in *S.cerevisiae* could alleviate the requirement for SR proteins in this organism.

SR proteins were independently discovered by a number of groups taking very different approaches. Splicing factor 2/alternative splicing factor (SF2/ASF) was the first SR protein to be identified. SF2 and ASF, which are identical, were independently isolated. SF2 was isolated from HeLa cell nuclear extract as a factor required to reconstitute splicing in an S100 splicing-deficient HeLa cell extract

(Krainer and Maniatis, 1985; Krainer et al., 1990a; 1991). ASF was isolated based on an activity that switched SV40 T/t 5' splice site usage *in vitro* (Fu and Manley, 1987; Ge and Manley, 1990; Ge et al., 1991). In another approach, monoclonal antibodies that inhibit the splicing reaction led to the identification of SC35 and 9G8 (Fu and Maniatis, 1990) (Cavaloc et al., 1994). The human SR protein family currently contains 11 known members. They each share the following characteristics: (1) They contain a shared phosphoepitope recognised by the monoclonal antibody mAb104 (Roth et al., 1991). (2) They copurify in a two-step salt precipitation procedure (soluble in 65% ammonium sulfate and precipitated in 20mM $MgCl_2$) (Zahler et al., 1992). (3) They can complement splicing-deficient cytoplasmic S100 extracts indicating a degree of overlapping function in splicing. (4) Their sizes on SDS-PAGE are conserved from *Drosophila* to man. (5) They are characterised by a modular domain structure, comprising one or two RNA-recognition motifs (RRMs) at their N-termini and a variable-length arginine/serine-rich domain at their C-termini (the RS domain).

1.8 The structural organisation of SR proteins

The RNA-recognition motifs (RRMs)

The RNA recognition motif (RRM) is a conserved, modular RNA binding domain of approximately 80 amino acids in length and contains two small highly conserved sequence elements, the RNP-1 octamer and the RNP-2 hexamer (Nagai et al., 1990; Hoffman et al., 1991). The RRM motif is present in one or more copies in many RNA-binding proteins involved in pre-mRNA and pre-rRNA processing (Kenan et

al., 1991; Birney et al., 1993). SR proteins contain one or two RRMs, however, in those SR proteins that contain two RRMs, the second RRM is atypical and always contains a heptapeptide motif, SWQDLKD, that is a signature of this domain (Birney et al., 1993). Mutation of the RNP-2 or RNP-1 motifs of SF2/ASF inhibits binding to RNA and results in decreased activity in *in vitro* splicing assays (Caceres and Krainer, 1993). The RRMs can bind RNA in a sequence specific manner in the absence of the RS domain (Zuo and Manley, 1993; Tacke and Manley, 1995b; 1999; van der Houven van Oordt et al., 2000). SR proteins recognise a vast array of RNA sequences. Although SR proteins do display specificities, the consensus sequences they recognise are degenerate and the binding site of one SR protein may also act as the binding site for another (Tacke and Manley, 1995a; 1999; Liu et al., 1998; Cavaloc et al., 1999).

The RS domain

The RS domain consists of simple repeats of arginine and serine, occasionally interrupted by other amino acids, and its length and sequence are highly conserved for individual SR proteins in different species (Zahler et al., 1992; Birney et al., 1993). The RS domain is also present in a family of related splicing factors called SR-like or SR-related proteins (reviewed by (Blencowe et al., 1999)). The RS domain can be functionally exchanged between SR proteins and can function when fused to a heterologous RNA-binding domain (Chandler et al., 1997; Graveley and Maniatis, 1998; Wang et al., 1998b).

The RS domains of SR proteins function as a protein-protein interaction domain. SR proteins were initially shown to interact with one another, with the splicing regulators Tra and Tra2, and with other components of the splicing

machinery that contain RS domains such as U1 snRNP-associated 70 kDa protein (U170K) and U2AF35 (Amrein et al., 1994; Wu and Maniatis, 1993; Kohtz et al., 1994). While the RS domains have been shown to be sufficient to mediate certain protein interactions, the RS domain of SF2/ASF is not sufficient to interact with U170K (Xiao and Manley, 1997). In addition, when artificially tethered to the pre-mRNA, the RS domains of several human SR proteins are sufficient to activate enhancer-dependent splicing, an activity that presumably requires protein interactions (Graveley and Maniatis, 1998).

The RS domain contacts the pre-mRNA at several stages during spliceosome assembly suggesting an additional role to mediating protein-protein interactions. RNA-protein cross-linking procedures demonstrate two RS domains, one from U2AF65 (Valcarcel et al., 1996) and a second from an SR protein, are required for prespliceosome assembly (Shen et al., 2004). These two RS domains contact the branchpoint sequentially: first the RS domain from the polypyrimidine-tract bound U2AF65 contacts the branchpoint in the E complex and subsequently, the RS domain from the SR protein contacts the branchpoint in the prespliceosomal A complex. A third RS domain, provided by another SR protein, is required for mature spliceosome assembly and contacts the 5' splice site in the B complex (Shen and Green, 2004). The RS domain-splicing signal interactions may promote (or stabilise) base-pairing between the U snRNA and pre-mRNA substrate, thereby enhancing splicing (Valcarcel et al., 1996; Shen et al., 2004; Shen and Green, 2004; Hertel and Graveley, 2005).

The RS domain of SF2/ASF is not required for *in vitro* splicing of all pre-mRNAs. Specifically the RS domain is dispensable for the processing of several constitutively spliced and enhancer dependent pre-mRNAs with strong splice sites

(Zhu and Krainer, 2000). In contrast the RS domain is required for splicing of an intron with a weakened polypyrimidine tract that also requires U2AF35. This suggests the existence of both RS domain-dependent and independent activities of SR proteins.

1.9 Role of SR proteins in constitutive splicing

In cellular extracts lacking SR proteins, such as S100 cytosolic extracts (Krainer et al., 1990b) no specific splicing complex could be detected, indicating that SR proteins act early in spliceosome assembly. In addition SR proteins can commit pre-mRNA to the splicing pathway (Fu, 1993). The binding of SR proteins to pre-mRNA clearly provides a kinetic advantage for assembly into these earliest detectable splicing complexes. SF2/ASF has been shown to bind RNAs containing wild-type, but not mutant 5' splice sites (Zuo and Manley, 1994). Furthermore, SF2/ASF cooperates with U1 snRNP in binding to and defining a functional 5' splice site by interacting with the U1 snRNP-associated 70 kDa protein (U170K) (Eperon et al., 1993; Wu and Maniatis, 1993; Kohtz et al., 1994; Jamison et al., 1995). The binding of SF2/ASF to the 5' splice site before U1 snRNP addition is prerequisite for complex formation. The observation that SR proteins and U1 snRNP associate with pol II led to the proposal that SR proteins promote cotranscriptional spliceosome assembly (Das et al., 2007).

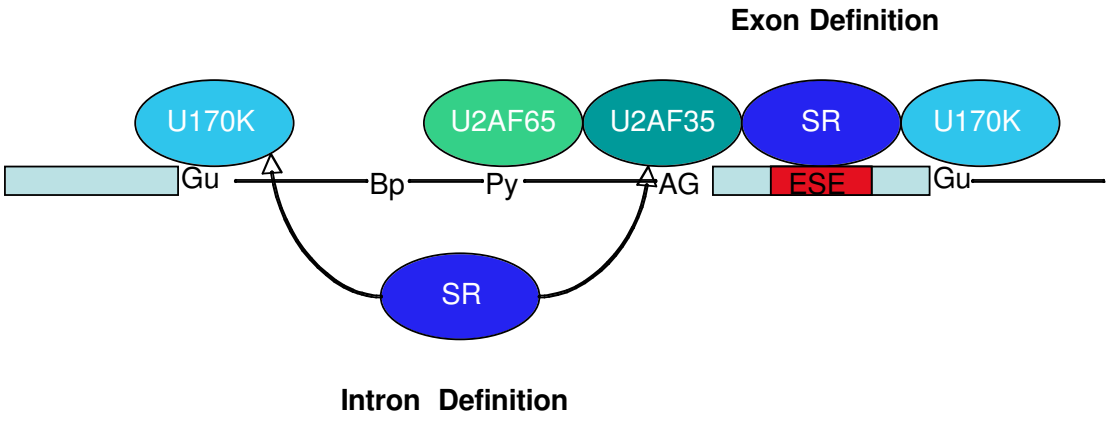


Figure 1.3: The exon-dependent and –independent functions of SR proteins in pre-spliceosome assembly. SR proteins can promote both the recognition of 5' and 3' splice sites as well as communication between splice sites by exon definition or intron bridging interactions. Arrows indicate RS domain-mediated interactions.

Bridging 5' and 3' splice sites

SR proteins directly promote E complex formation by stabilising U1 snRNP bound at the 5' splice site (Kohtz et al., 1994) and U2AF bound to the polypyrimidine tract (Staknis and Reed, 1994). The SR proteins also bridge the 5' and 3' splice sites by promoting RS-mediated interactions with U170K at the 5'splice site and with the small subunit of the U2 snRNP auxiliary factor (U2AF35) at the 3' splice site (Wu and Maniatis, 1993; Kohtz et al., 1994) (Figure 1.3). Although not all SR proteins have been tested for this interaction, at least one - SRp54 – does not interact with U2AF35 but instead U2AF65 (Zhang and Wu, 1996). Thus, a network of protein-protein interactions builds a bridge between the 5' and 3' splice sites, effectively looping out the intron during prespliceosomal assembly, this is thought to play a key role in intron definition. This network of interactions provides a mechanistic explanation for earlier observations that SC35 is required to mediate interactions between U1 and U2 snRNP at the 3' splice site (Fu and Maniatis, 1992). This model is also supported by bimolecular splicing experiments demonstrating that 5' and 3' splice site complexes on separate transcripts can be *trans*-spliced, and that SR proteins appear to play an important role in mediating splice site interactions in *trans* (Bruzik and Maniatis, 1995; Chiara and Reed, 1995).

Network interactions across the exon

SR proteins and SR related proteins assemble on discrete sequences within exons, termed exonic splicing enhancers (ESEs), to promote both constitutive and alternative splicing by forming networks of interactions with each other, as well as with snRNP-

associated, SR related proteins (reviewed by (Blencowe, 2000)). The bound SR proteins can interact with U2AF35 to stimulate and/or stabilise recruitment of U2AF recruitment to the polypyrimidine tract and activate adjacent 3' splice sites (U2AF recruitment model) (Wang et al., 1995; Zuo and Maniatis, 1996; Graveley et al., 2001). The recruitment model does not seem to be generally applicable to all enhancer-dependent splice sites (Kan and Green, 1999; Li and Blencowe, 1999). For certain pre-mRNA substrates, the presence of an ESE-bound SR protein does not act to promote recruitment of U2AF to the polypyrimidine tract, rather it helps to antagonise the negative activity of hnRNP proteins recognising exonic splicing silencer (ESS) elements (Eperon et al., 2000). A role for two SR-related nuclear matrix proteins (SRm160/300) in promoting enhancer dependent splicing has also been proposed (Eldridge et al., 1999). ESE bound SR proteins might interact with the splicing coactivator SRm160 and establish a set of interactions with one or more snRNP components.

Downstream 5' splice sites are able to stimulate the splicing efficiency of upstream introns (Robberson et al., 1990; Kuo et al., 1991) and even promote splicing in *trans* (Chiara and Reed, 1995). It was demonstrated that the effect of the downstream 5' splice site is due to a network of interactions spanning the exon involving U1 snRNP binding to the downstream 5' splice site and U2AF65 binding to the polypyrimidine tract in the upstream intron (Hoffman and Grabowski, 1992).

The exon definition model (Berget, 1995) proposes that interactions between components bound to the 5' and 3' splice sites flanking an exon serve to distinguish exons from introns. SR proteins have been proposed to participate in this process where they simultaneously interact with U2AF35 bound to the upstream 3' splice site and U170K bound to the downsteam 5' splice site (Wu and Maniatis, 1993). It is

thought that the majority of constitutively spliced exons are defined by this mechanism (Reed, 1996). In support of this model a number of SR protein binding sites that function as constitutive splicing enhancers have been identified in constitutive exons (Mayeda et al., 1999; Schaal and Maniatis, 1999).

In summary, SR proteins are involved in three processes during the early stage of spliceosome assembly (Figure 1.3). (1) SR proteins cooperate with U1 snRNP in defining a functional 5' splice site. (2) SR proteins interact with the U2AF hetrodimer to promote and/or stabilise complex assembly at the 3' splice site. (3) SR proteins bridge factors assembled on both 5' and 3' splice sites to form stable commitment complexes. These complexes assemble both across the intron and the exon. Additionally, SR proteins function at later stages of the splicing reaction facilitating the recruitment of the U4/U6.U5 tri-snRNP complex (Roscigno and Garcia-Blanco, 1995).

1.10 Actions of SR proteins and hnRNP A/B proteins in splice site selection

The first SR proteins to be identified had similar effects on 5' splice site selection. Increased concentrations of the proteins resulted in the selection of intron-proximal 5' splice sites in pre-mRNAs that contain two or more alternative 5' splice sites (Fu and Manley, 1987; Krainer et al., 1990c; Ge and Manley, 1990). An excess of hnRNP A/B proteins had the opposite effect, promoting the selection of intron-distal 5' splice sites. These effects have been observed with different pre-mRNA substrates both *in vitro* and *in vivo* (Mayeda and Krainer, 1992; Mayeda et al., 1993; Caceres et al., 1994; Yang et al., 1994; Wang and Manley, 1995). Individual SR proteins can

sometimes have opposite effects on alternative splice site selection, as in the case of the antagonistic effects of SF2/ASF and SC35 in the regulation of β-tropomyosin (Gallego et al., 1997) and of SF2/ASF and SRp20 on the regulation of SRp20 pre-mRNA alternative splicing (Jumaa and Nielsen, 1997). The relative abundance of SR and hnRNP A/B proteins could be important in regulating the patterns of alternative splicing in a tissue specific or developmentally regulated manner. There are tissue-specific variations in the total and relative amounts of SR proteins (Zahler et al., 1993). The molar ratio of SF2/ASF to its antagonist hnRNP A1 varies considerably in different rat tissues (Hanamura et al., 1998). All SR genes have alternative spliced forms that are expected to be degraded by nonsense mediated mRNA decay due to the alternative splicing of highly or ultraconserved elements. Thus SR proteins can auto regulate their own expression by coupling alternative splicing with decay (Lareau et al., 2007)

1.11 Functional redundancy among SR proteins

SR proteins are functionally redundant in the splicing of some introns. However, several differences in the ability of these proteins to regulate alternative splicing, as well as the ability of individual SR proteins to commit different pre-mRNAs to the splicing pathway suggested that individual SR proteins have unique functions in splicing regulation (Fu, 1993; Caceres et al., 1994; Wang and Manley, 1995; Chandler et al., 1997). Genetic analyses of SR proteins have demonstrated that not all SR proteins are functionally redundant. SF2/ASF is essential for cell viability in the DT40 chicken cell line, and its depletion cannot be rescued by over expression of other SR proteins (Wang et al., 1998b). Furthermore, SF2/ASF has been shown to

play an essential role in regulating an alternative splicing pathway that is crucial for postnatal heart remodelling in the mouse (Xu et al., 2005). SRp20 is essential for mouse development (Jumaa et al., 1999) and conditional deletions of the SR protein SC35 in the thymus causes a defect in T cell maturation (Wang et al., 2001). RNA interference (RNAi) experiments with *Caenorhabditis elegans* SR proteins showed that, whereas the ortholog of the mammalian SF2/ASF (CeSF2/ASF) is an essential gene, functional knockouts of other SR genes resulted in no obvious phenotype, which is indicative of functional redundancy. Instead lethality was only seen when combinations of these SR proteins were targeted simultaneously (Kawano et al., 2000; Longman et al., 2000). In *Drosophila*, the B52 gene, a homolog of human SRp55, is essential for development, although several genes show proper pre-mRNA splicing in the arrested larvae (Ring and Lis, 1994; Peng and Mount, 1995). It was later found that other SR proteins complement the loss of B52 in most tissues (Hoffman and Lis, 2000). However in the brain, where B52 is the predominant SR protein, the levels of SR proteins are not sufficient to compensate for loss of B52 in the null mutant. These results further indicate that the requirement for a particular SR protein may be due to specific functions in the tissue or developmental stage in which a particular SR protein is predominant.

1.12 Transcriptional regulation of alternative splicing

The regulation of alternative splicing depends not only on the interaction of splicing factors with splicing regulatory elements in the pre-mRNA, but also on promoter type and the recruitment of transcription factors and coactivators (Cramer et al., 1997;

Cramer et al., 1999; Kadener et al., 2001; Nogues et al., 2002; Auboeuf et al., 2004; Kornblihtt, 2005). The impact of steroid hormone-mediated transcription on RNA processing was investigated with reporter genes subject to alternative splicing driven by steroid-sensitive promoters (Auboeuf et al., 2002). Activated steroid receptors may bind to target DNA response elements and promote the recruitment of coregulators that are involved in both transcription and splicing. Depending on the promoter and cellular context, the same transcriptional factor could recruit different coregulators, thereby mediating different effects on transcription and splicing regulation.

The rate and pausing of transcription elongation has also been shown to regulate alternative splicing (Roberts et al., 1998; de la Mata et al., 2003; Howe et al., 2003). For instance, a slow pol II, and/or the presence of internal transcription pause sites, results in inclusion of the alternative exon harbouring a weak 3' splice site. By contrast, when the same pre-mRNA is transcribed by a highly processive pol II, the weak alternative 3' splice site is unable to compete with the stronger downstream 3' splice site, which results in skipping of the alternative exon.

Changes in chromatin structure have also been shown to affect splicing. For example, trichostatin A, an inhibitor of histone deacetylation, favours exon skipping of alternative exons, presumably because hyperacetylation of core histones facilitates the passage of the transcribing polymerase (Nogues et al., 2002). SWI/SNF is a chromatin-remodelling factor that plays a role in alternative splicing (Batsche et al., 2006) but, surprisingly, the mechanism of action is independent of its role in chromatin remodelling. Instead it forms macromolecular complexes with pol II at sites of variable exons resulting in the stalling of pol II that could favour the inclusion of variable exons in the mature mRNA.

1.13 HCC1

U2AF65 contains an N-terminal RS domain and three RRMs, all of which have been implicated in high-affinity binding to the polypyrimidine tract (Zamore et al., 1992). RRMs 1 and 2 of U2AF65 exhibit a canonical RRM-fold and bind RNA *in vitro* (Ito et al., 1999). The N-terminal amino acids 85-112 of U2AF65 (which does not comprise its RS domain) are required for the interaction with U2AF35 (Zhang et al., 1992). The atypical third RRM mediates the interaction with SF1 and could have a dual function in both RNA and protein binding (Berglund et al., 1998; Rain et al., 1998). This unusual RRM-like domain, called UHM for U2AF homology motif, is present in many other splicing proteins (Kielkopf et al., 2004).

PUF60 (poly (U)-binding protein factor-60 kDa) was first isolated as a protein closely related to U2AF65 that was required for efficient reconstitution of splicing *in vitro* (Page-McCaw et al., 1999). The homology between PUF60 and U2AF65 extends across their entire length, except for the N-terminus where PUF60 lacks a recognisable RS domain. CAPERα (also called HCC1.3) and CAPERβ are the most recently characterised proteins related to U2AF65 (Dowhan et al., 2005). Both have a domain structure similar to U2AF65, except for the C-terminus of CAPERβ, which lacks the UHM domain (reviewed by (Mollet et al., 2006)) (Figure 1.4).

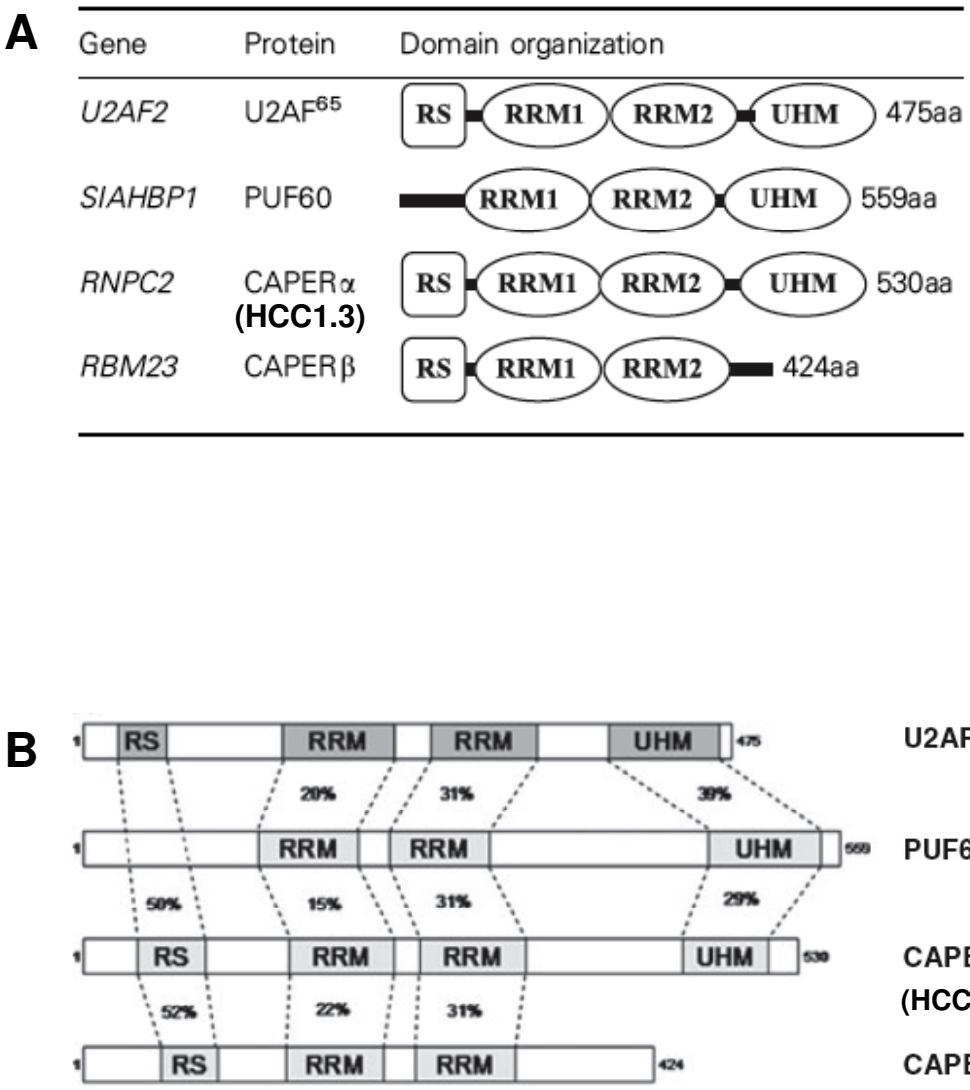


Figure 1.4: (A) Domain organisation of U2AF65 and U2AF65-related proteins. The gene names have been included. **(B) A schematic alignment of human proteins related to U2AF65.** The putative functional domains in each protein are aligned with U2AF65, and the similarity (% identity) of these domains in relation to U2AF65 is indicated (Mollet et al., 2006)

Although it remains to be determined whether U2AF65 performs other functions in the cell in addition to its fundamental role in pre-mRNA splicing, the U2AF65-related proteins are clearly implicated in both splicing and transcription. HCC1 was originally identified and cloned as an autoantigen from a patient with hepatocellular carcinoma (Imai et al., 1993). It comprises two related proteins, most likely generated by alternative splicing termed, HCC1.3 and HCC1.4, which differ by 6 amino acids. CAPER (HCC1.3) was independently identified as a protein that interacts with the estrogen receptor and stimulates its transcriptional activity, and was also purified as a spliceosome component capable of affecting the splicing reaction (Hartmuth et al., 2002; Jung et al., 2002; Rappsilber et al., 2002; Auboeuf et al., 2004). More recently, an additional related protein was identified and termed CAPERβ. Both CAPER (renamed CAPERα) and CAPERβ were shown to regulate transcription and alternative splicing in a steroid hormone-dependent manner (Dowhan et al., 2005). In response to steroid hormones CAPER (HCC1) proteins may interact with promoter-bound transcription factors resulting in the stimulation of transcription and its incorporation into the pre-initiation complex. CAPER proteins would then have direct access to the nascent RNA transcript allowing for interactions with splicing factors required for the early recognition of the 3' splice site thus influencing commitment to splicing (Dowhan et al., 2005). In support of this view HCC1.4 has been shown to interact with the SR-related protein, SRrp53, which regulates alternative splicing, possibly through activating weak 3' splice sites (Cazalla et al., 2005).

1.14 Nuclear organisation

Biochemical, genetic and molecular experiments have led to an overwhelming amount of information about the molecular mechanisms of transcription and pre-mRNA splicing. In contrast little is known about how these processes are integrated into the structural framework of the cell nucleus and how they are spatially and temporally co-ordinated within the three-dimensional confines of the nucleus (reviewed by (Lamond and Earnshaw, 1998; Misteli and Spector, 1998; Dundr and Misteli, 2001a)).

Genetically encoded fluorescent proteins have transformed studies in cell biology. The extensive mutagenesis of green fluorescent protein, from the jellyfish *Aequorea Victoria* (Chalfie et al., 1994), combined with the cloning of new fluorescent protein variants from corals, has yielded fluorescent proteins that emit light from the blue to the red end of the visible spectrum (Matz et al., 1999; 2002; Patterson et al., 2001; Zhang et al., 2002)) The emergence of these genetically encoded fluorescent tags allows the visualization and quantitative analysis of chromatin, mRNA and proteins in living cells (Misteli, 2001a; 2007; Lanctot et al., 2007; Rodriguez et al., 2007). These studies have revealed two fundamental aspects of nuclear architecture: first, time-resolved experiments revealed that the nucleus is a highly dynamic organelle and secondly, many nuclear factors are localised in distinct structures, such as speckles, paraspeckles, nucleoli, cajal bodies, gems and promyelocytic leukaemia bodies (Lamond and Sleeman, 2003; Handwerger and Gall, 2006). In this thesis I will focus on the speckles, also referred to as SC35 domain or splicing factor compartments (SFCs).

Evidence suggests that self-organisation plays a crucial role in generating nuclear architecture (Misteli, 2001b; 2007). Photobleaching experiments demonstrated that the steady-state accumulation of mobile nuclear components in compartments is produced by their transient interaction with locally immobilized binding sites (Misteli, 2001a). Furthermore the local concentration of factors created in these compartments, and the flux of components between compartments play key roles in regulating gene expression (Carmo-Fonseca, 2002).

1.15 Cell biology of splicing factors

Virtually all proteins involved in splicing are enriched in nuclear speckles, in addition to their diffuse distribution throughout the nucleoplasm (Spector et al., 1983; Lamond and Spector, 2003). These domains were described on the basis of their distinct morphology long before the discovery of pre-mRNA splicing (Beck, 1961). The domains can be resolved by electron microscopy into two distinct structures. One is composed of clusters of electron-dense granules of about 20nm in diameter situated between chromatin regions and, therefore, termed interchromaatin granule clusters (IGCs) (Swift, 1959). The second is composed of perichromatin fibrils (PFs) that radiate from the periphery of the clusters of IGCs and are also found distributed throughput the nucleoplasmic space (Monneron and Bernhard, 1969).

Several lines of evidence point to speckles functioning as storage/assembly/modification compartments that can supply splicing factors to active transcription sites (Misteli, 2000). Speckles are often observed close to highly active transcription sites and specific highly active genes have been shown to localise preferentially with the periphery of speckles (Huang and Spector, 1991; Xing et al.,

1993; Xing et al., 1995; Smith et al., 1999; Johnson et al., 2000; Moen, Jr. et al., 2004). Nucleotide incorporation experiments demonstrated that active sites of transcription are associated with PF rather than within IGCs (Fakan and Bernhard, 1971; Fakan and Nobis, 1978; Cmarko et al., 1999). Live cell studies show the recruitment of splicing factors from speckles to active sites of transcription (Misteli et al., 1997) and conversely, speckles become rounded up and enlarged upon disruption of transcription or splicing (Spector et al., 1983; O'Keefe et al., 1994). Furthermore, upon expression of intron-containing genes or viral genes, splicing factors are redistributed from speckles to the new transcription sites (Jimenez-Garcia and Spector, 1993; Bridge et al., 1995; Huang and Spector, 1996; Misteli et al., 1997). While these observations suggest that splicing factors generally move towards a gene, it is likely that pre-mRNA also moves towards speckles (Xing et al., 1993; Melcak et al., 2000).

The fact that individual splicing factors undergo redistribution upon gene activation implies that their subnuclear distribution is regulated. In the *Drosophila melanogaster* Tra protein the RS domain is both necessary and sufficient for localisation to nuclear speckles (Li and Bingham, 1991). However the RS domain is not necessary in SF2/ASF and U2AF65 for a speckled localisation (Caceres et al., 1997; Gama-Carvalho et al., 1997). Although the RS domain of SF2/ASF is a nuclear localisation signal, subcellular targeting to the speckles requires at least two of the three domains of SF2/ASF, which contain additive or redundant signals. In contrast, in two SR proteins that have a single RRM, SRp20 and SC35, the RS domain is necessary and sufficient for proper localisation (Caceres et al., 1997).

1.16 Composition of speckles

Many pre-mRNA splicing factors – including snRNPs and SR proteins – have been localised to nuclear speckles. In addition, several kinases (Colwill et al., 1996; Ko et al., 2001; Kojima et al., 2001; Sacco-Bubulya and Spector, 2002; Brede et al., 2002) and phosphatases (Trinkle-Mulcahy et al., 1999; 2001) that can phosphorylate/dephosphorylate components of the splicing machinery have also been localised to nuclear speckles. In an attempt to characterise in detail the protein composition of speckles, proteomic analysis of an enriched IGC fraction purified from mouse liver nuclei has been carried out – 136 known proteins, as well as numerous uncharacterised proteins have been identified (Mintz et al., 1999). In addition to pre-mRNA splicing factors, several other proteins such as RNA export factors (Zhou et al., 2000; Le Hir et al., 2001; Degot et al., 2004) transcription factors (Larsson et al., 1995; Zeng et al., 1997), subunits of pol II (Mortillaro et al., 1996), 3'-end RNA processing factors (Krause et al., 1994; Schul et al., 1998), translation factors (Li et al., 1999; Dostie et al., 2000), and structural proteins (Nakayasu and Ueda, 1984; Jagatheesan et al., 1999), such as lamin A and snRNP associated actin, have been localised to nuclear speckles. In addition to these factors, a population of poly (A) $^{+}$ RNA has been found associated with nuclear speckles (Carter et al., 1991; Visa et al., 1993; Huang et al., 1994). These RNAs may represent species with a specific nuclear function. For example they may act as structural RNAs involved in the organisation of speckles.

1.17 Nuclear speckles and the cell cycle

At the onset of mitosis, mammalian cell nuclei undergo dramatic structural and functional alteration such as chromatin condensation, inactivation of the transcription machinery, nuclear envelope break down, and disassembly of nuclear compartments. Nuclear speckles disassemble during mitosis, and their constituents are diffusely distributed throughout the cytoplasm, later organising into cytoplasmic structures called mitotic interchromatin granules (MIGs) (Reuter et al., 1985; Spector and Smith, 1986; Verheijen et al., 1986; Leser et al., 1989; Ferreira et al., 1994; Thiry, 1995). Early studies on the behaviour of nuclear speckles through the cell cycle described MIGs as the mitotic equivalent of interphase nuclear speckles based on their similar granular structure and composition. MIGs disappear concomitant with nuclear entry of pre-mRNA processing factors at telophase, showing that these factors are recycled from the cytoplasm into daughter nuclei (Prasanth et al., 2003). Splicing factors are competent for pre-mRNA processing immediately after their entry into daughter nuclei (Prasanth et al., 2003), supporting the possibility that MIGs might be responsible for splicing-factor modification, allowing for the immediate targeting of pre-mRNA processing factors to transcription sites in telophase nuclei. Telophase splicing factors do not immediately localise to nuclear speckles upon nuclear entry. SR proteins factors initially localise around nucleolar organising regions before the establishment of nuclear speckles, whereas snRNPs localise in polar regions of daughter nuclei (Bubulya et al., 2004). The concentration of SR proteins in a region of the nucleus away from other splicing factors could increase the probability of RS-domain mediated interactions, thus facilitating the intermolecular associations important for subsequent association with transcription sites and nuclear speckles.

1.18 Nucleo-cytoplasmic shuttling of SR proteins

Whereas some human SR proteins are confined to the nucleus, three of them – SF2/ASF, SRp20, and 9G8 - shuttle rapidly and continuously between the nucleus and the cytoplasm (Caceres et al., 1998) an activity reminiscent of hnRNP family members (Pinol-Roma and Dreyfuss, 1992). Chimeric constructs between a shuttling protein SF2/ASF, and two non-shuttling proteins, SRp40 and SC35, demonstrated that the RS domain of SF2/ASF is required for nucleo-cytoplasmic shuttling. The RS domain of SF2/ASF conferred the ability to shuttle to a nonshuttling protein, SC35, when substituted for the natural RS domain of this protein. Conversely, replacing the RS domain of SF2/ASF with the RS domain of SRp40 converted a shuttling protein into one that remained confined to the nucleus (Cazalla et al., 2002). The RS domain also plays a role in nuclear localisation and is necessary for their interaction with transportin-SR, a nuclear import receptor specific for SR proteins (Caceres et al., 1997; Kataoka et al., 1999).

This subset of shuttling SR proteins are involved in several post-splicing activities. Two shuttling SR proteins, SRp20 and 9G8, have been shown to promote mRNA export of intronless RNAs and to act as adapter proteins for TAP-dependent mRNA export (Huang and Steitz, 2001; Huang et al., 2003). SF2/ASF has been shown to control the cytoplasmic stability of a specific mRNA (Lemaire et al., 2002). Furthermore, shuttling SR proteins associate with translating ribosomes and enhance translation of reporter mRNAs both *in vivo* and *in vitro* (Sanford et al., 2004). It has also been demonstrated that SR proteins are involved in nonsense-mediated mRNA decay, although this activity does not correlate with their ability to shuttle (Zhang and Krainer, 2004).

1.19 Phosphorylation of SR proteins

RS domains are heavily phosphorylated *in vitro* and *in vivo* and several kinase activities have been reported to phosphorylate SR proteins (Woppmann et al., 1993; Gui et al., 1994; Colwill et al., 1996; Nikolakaki et al., 1996; Rossi et al., 1996; Wang et al., 1998a). Functionally, phosphorylation of the RS domain contributes to RNA-binding specificity (Tacke et al., 1997), modifies protein-protein interactions (Xiao and Manley, 1997; Wang et al., 1998a) and plays a role in regulating alternative splicing, nuclear import and export, and translation (Caceres et al., 1998; Lai et al., 2001; Blaustein et al., 2005; Sanford et al., 2005). *In vitro* studies demonstrate that a cycle of phosphorylation and dephosphorylation is essential for the splicing reaction to take place (Mermoud et al., 1992; 1994; Tazi et al., 1993; Cao et al., 1997). It has been observed that certain pre-mRNA splicing factors must be phosphorylated before they can be incorporated into functional spliceosomes and that dephosphorlation occurs as the splicing reaction takes place.

The effect of phosphorylation on splicing factor function is mirrored by its effects on their localisation. Three splicing factor-specific kinases, SRPK-1, SRPK-2 and Clk/Sty cause the release of splicing factors from nuclear speckles (Gui et al., 1994; Colwill et al., 1996; Wang et al., 1998a). Clk/Sty may also be involved in the release of SR proteins from nucleolar organising regions in telophase cells (Bubulya et al., 2004). On the other hand, a serine/threonine protein phophatase 1 activity has been identified which has the opposite effect on splicing factor distribution (Misteli and Spector, 1996).

These observations have led to the following model for the control of splicing factor distribution in the nucleus. Functionally inactive pre-mRNA splicing factors

localise in IGCs. Phosphorylation of the factors allows them to be released from IGCs and to be recruited, possibly in a complex with other processing components, to transcription sites, where the spliceosome forms on nascent RNAs. During splicing or after the splicing reaction, the factors are dephosphorylated and the spliceosomes are eventually disassembled. The dephosphorylated factors can now reassociate with the IGCs or travel to the cytoplasm and enter a new round of phosphorylation and dephosphorylaion.

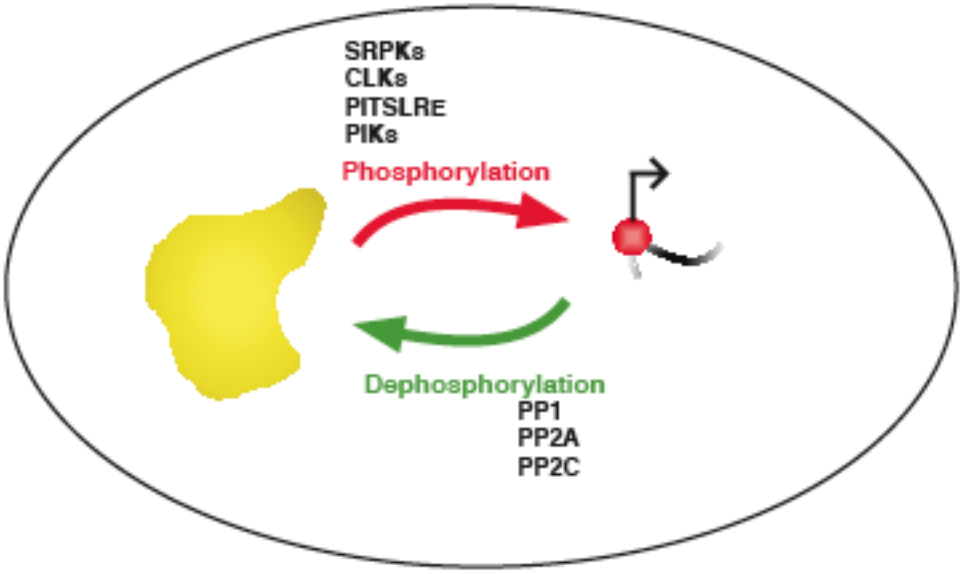


Figure 1.5: Role of phosphorylation in regulating the nuclear properties of splicing factors. The phosphorylation of SR proteins is a pre-requisite for their release from nuclear speckles. Numerous kinases that localize to nuclear speckles and potentially mediate release of splicing factors have been identified. Progression through the splicing reaction, resoltion of the spliceosome and reassociation of splicing factors with nuclear speckles requires multiple dephosphorylation steps (Misteli,2000).

1.20 Fluorescent Resonance Energy Transfer (FRET)

Light microscopy has initiated our understanding of cellular structure and the associated function. Approaches are being used to monitor the co-localisation of proteins with different subcellular compartments, providing critical information about cell physiology. The problem is that the detection of protein co-localisation alone cannot distinguish proteins with overlapping distribution from those proteins that are interacting in significant ways. Molecular biology studies over the past few decades have shown that cellular events, such as signal transduction and gene expression, require the assembly of proteins into specific macromolecular complexes. Traditional biochemical methods did not provide information about the interactions of these protein partners in their natural environment. Such progress has now been made in fluorescence microscopy in both the development of new methods and the engineering of fluorescent probes that the biology of the cell can now be investigated at macromolecular levels in biological space and time.

Fluorescence resonance energy transfer (FRET) is a quantum mechanical phenomenon that occurs between a fluorescent donor and a fluorescent acceptor that are in molecular proximity of each other if the emission spectrum of the donor overlaps the excitation spectrum of the acceptor (reviewed by (Day et al., 2001a; Wouters et al., 2001; Chen et al., 2003)). Under these conditions, energy is transferred non-radiatively from the donor to the acceptor (Figure 1.5). The efficiency of energy transfer is dependent on the distance between the two fluorophores, the relative orientation of the donor and acceptor and the fraction of interacting fluorophores (Patterson et al., 2000). Thus, FRET can be applied to biological systems to glean

information about molecular structure, and the magnitude and dynamics of interactions between donor- and acceptor-labelled molecules (Hoppe et al., 2002).

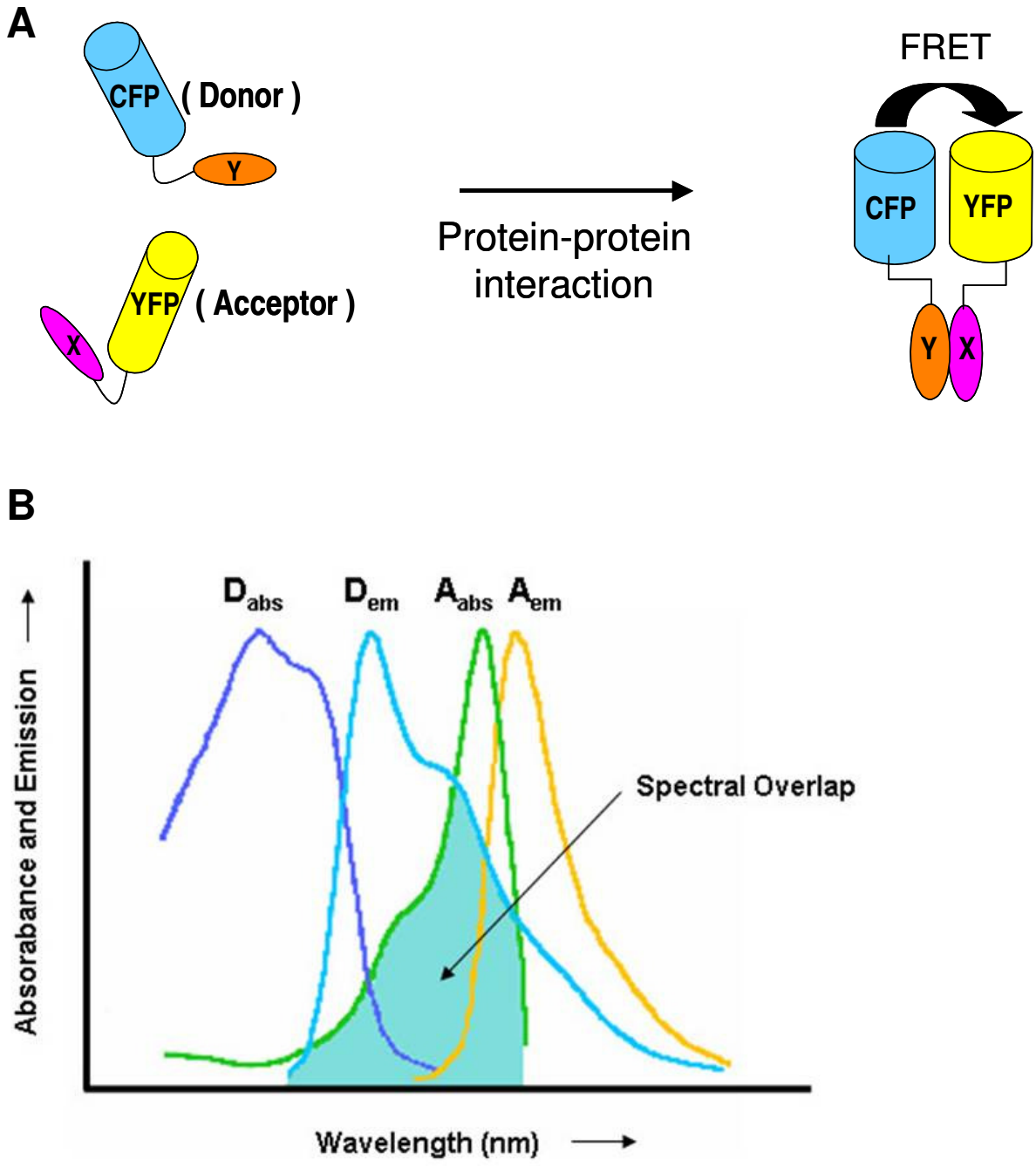


Figure 1.6: Principles of fluorescence resonance energy transfer (FRET). (A) An intermolecular FRET-based reporter consists of two different proteins (X and Y) that are labelled with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively, which interact and bring the fluorophores into close proximity, thereby increasing FRET efficiency (Zhang et al., 2002). (B) Excitation and emission of a commonly used FRET pair. The schematic depicts simplified absorbance and emission spectrum of CFP (donor; D) and YFP (acceptor; A). Overlap between CFP emission and YFP absorbtion (shaded region) is a prerequisite for FRET. Donor absorbance (D_{abs}), Donor emission (D_{em}), Acceptor emission (A_{em}), Acceptor absorbance (A_{abs}) (Bhat et al., 2006).

Excitation of a donor fluorophore in a FRET pair leads to quenching of the donor emission and to an increased, sensitised, acceptor emission. Intensity-based FRET detection methods include monitoring the donor intensity (with or without acceptor photobleaching), the sensitised acceptor emission or the ratio between the donor and acceptor intensity. Methods that are based on fluorescent-decay kinetics include determining the rate of donor photobleaching, the appearance of new components in the acceptor decay kinetics or the decrease of donor fluorescent lifetime. When a fluorophore absorbs a photon it enters an excited state and returns to the ground state by emitting a lower energy photon, with the energy difference between absorbed and emitted photon transferred to the environment. The time taken for the energised electron to return to the ground state occurs on the nanosecond time scale and is referred to as the fluorescent lifetime (Figure 1.6). Fluorescent Lifetime Imaging Microscopy (FLIM) allows the population of interacting protein species to be determined on a point-by-point basis at each resolved voxel in the cell (Becker et al., 2001).

There are two complementary techniques of lifetime measurement: the time domain and the frequency domain. In the time domain, a short pulse of light excites the sample, and the subsequent fluorescence emission is recorded as a function of time. This usually occurs on the nanosecond timescale. In the frequency domain, the sample is excited by a modulated source of light. The fluorescence emited by the sample has a similar waveform, but is modulated and phase-shifted from the excitation curve. The lifetime can be calculated from the observed modulation and phase-shift.

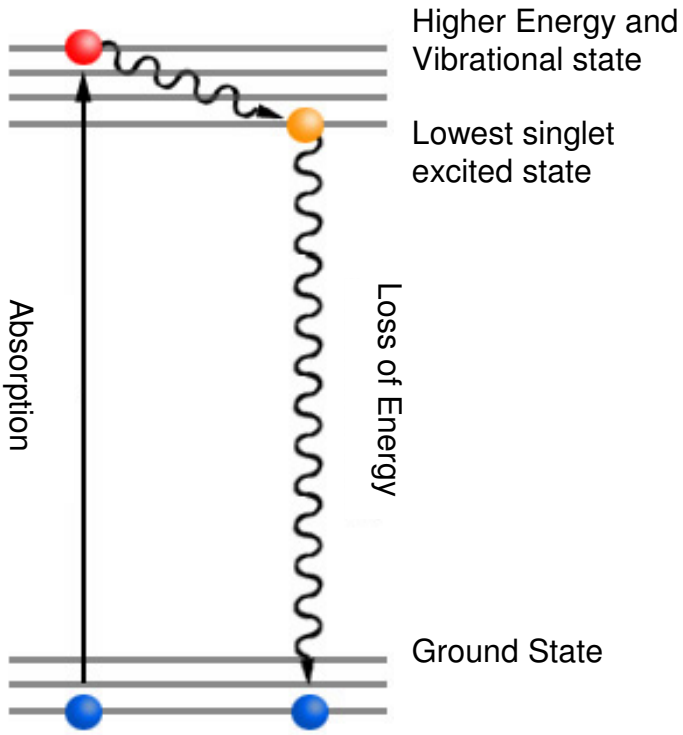


Figure 1.7: Principles of fluorescence. Within femtoseconds after light excites the fluorophore at an appropriate wavelength, its electron jump from the ground state to a higher vibrational state. Within picoseconds these electrons decay back to the lowest excited state, and then decay more slowly (nanoseconds) back to the ground state with the emission of a photon whose wavelength is longer than the exciting wavelength (mekentosj.com/science/fret/fluorescence.html).

1.21 Applications of FRET

The earliest FRET reporters utilised in living cells consisted of BFP and GFP fused with a protease-sensitive linker. Proteolysis disrupts FRET by separating the donor and acceptor units (Mitra et al., 1996). FRET reporters have been applied to a range of cell biological phenomena. CAMeleons are FRET reporters used to measure changes in intracellular Ca^{2+} concentration (Miyawaki et al., 1997). The binding of Ca^{2+} results in conformational changes leading to a large increase in FRET. Beyond intracellular ion sensing, FRET constructs have been used to visualise the behaviour of many other signalling molecules and proteins in cells. For example, indicators for kinase activity (Kurokawa et al., 2001; Ting et al., 2001; Zhang et al., 2001; Sato et al., 2002), cGMP (Sato et al., 2000; Honda et al., 2001), Ras and Rap1 activity (Mochizuki et al., 2001), and Ran activity (Kalab et al., 2002) have been constructed. FRET imaging can also be used to observe interactions between GFP fusion proteins and fluorescently labelled antibodies. For example, FLIM has been used to show autophophorylation of GFP-tagged PKC or ErbB1 by detecting FRET between the GFP-tagged protein and a fluorescently labelled phosphorylation-site-specific antibody (Ng et al., 1999; Verveer et al., 2000b; Wouters and Bastiaens, 1999). A FRET-based reporter of membrane potential has also been developed (Sakai et al., 2001).

The attachment of donor and acceptor fluorophores to interacting protein partners allows interactions to be monitored in real-time in live cells. This technique has been applied to study a wide range of interactions, however, at this point in time there are relatively few studies of interactions between RNA processing factors. FRET has been utilised to address a proposed role for cajal bodies in snRNP assembly (Stanek and Neugebauer, 2004). The distribution of snRNP intermediates required for

U4/U6 snRNP assembly demonstrated that U4/U6 snRNP assembly occurs preferentially in cajal bodies. FRET has also been applied to study the interactions between U2AF35 and U2AF65 and revealed a novel U2AF35 self-interaction (Chusainow et al., 2005).

Protein-protein interactions can also be imaged in live cells by bimolecular fluorescence complementation (BiFC) assays, in which the potential partner proteins are fused to complementary fragments of fluorogenic reporters. The interaction of the protein partners allows the two fragments to reconstitute the fluorescence. The BiFC assay was applied to study complex formation between the splicing factor Y14 and nuclear export factor 1 (NXF1), which are functionally associated with nuclear mRNA (Schmidt et al., 2006) and showed that fluorescence accumulated in and around speckles. This led to the conclusion that a fraction of RNA, which remains in the nucleus for several hours despite its association with splicing and export factors, accumulates in speckles.

1.22 Conclusions

In vitro experiments have led to an overwhelming amount of information about the molecular mechanisms of transcription and pre- mRNA splicing. In contrast little is known about how these processes are integrated into the structural framework of the cell nucleus and how they are spatially and temporally co-ordinated within the three-dimensional confines of the nucleus. Colocalisation experiments have been used to determine which factors reside in the different subcompartments of the nucleus leading to insights into their function. However, FRET assays can be employed to determine whether direct protein-protein interactions occur in subcompartments allowing the visualisation of protein complexes in living cells.

Here I have employed FRET acceptor photobleaching and FLIM to map with nanometer resolution interactions between splicing factors within living human cells. This approach allows the study of interactions at the single cell level complementing previous biochemical data. I have utilised this approach to investigate the interactions of SR proteins with U2AF35 and U170K. These interactions have been extensively characterised *in vitro* and are proposed to play a crucial role in intron and exon definition. This provides a well defined model to test by FRET acceptor photobleaching and FLIM. The aims of this thesis are to determine whether interactions previously described *in vitro* occur within the nucleus of living cells and to map the locations of these interactions.

Splicing frequently occurs cotranscriptionally, therefore by inhibiting transcription for 2 hours the number of spliceable transcripts remaining in the cell is expected to decrease. Therefore by carrying out FRET experiments in the presence and absence of the transcription inhibitor DRB I aimed to determine whether the

interactions between splicing factors are dependent on splicing. The interactions of SR proteins with U170K and U2AF35 have been spatially mapped within live cell nuclei and occur in the presence of the pol II inhibitor, DRB, demonstrating that they are not exclusively cotranscriptional.

In addition I have characterized novel interactions of HCC1, a factor related to U2AF65, with both U2AF35 and U2AF65 suggesting a role for HCC1 in 3' splice site selection. FLIM microscopy showed the interactions between HCC1 and U2AF65 occurred more strongly in discrete domains within the nucleoplasm. In conclusion I have demonstrated different complexes of splicing factors show different patterns of interactions within live cell nuclei.

Materials and Methods

2.1 Plasmid Constructs

Human U170K and Luc7A were cloned by reverse transcription (RT-PCR) from total RNA from HeLa cells. Total RNA was prepared using TriReagent (Sigma) according to the manufacturer's specifications. 5 μg of total RNA was used for synthesis of first-strand cDNA with SuperScript^{TM}II RNase H^{-} reverse transcriptase (Invitrogen), following manufacturer's protocol. Oligo dT primers were used for the RT reaction and 10% of the cDNA obtained in each case was used for PCR amplification. Fragments corresponding to full length coding sequence of human U170K or Luc7A were amplified using specific primers that introduce EcoR1 and BamH1 restriction sites, ligated into the corresponding sites of ECFP-C1 or EYFP-C1 (clonetech). Alternatively Xba1 and BamH1 restriction sites were introduced for ligation of U170K into the mammalian expression vector pCG-T7. Human HCC1.4, SRp20, SC35 and SF2/ASFΔRS were cloned by using the cDNA cloned into the mammalian expression vector pCG-T7 as a template for PCR. Specific primers introduced BglII and BamH1 restriction sites for subsequent ligation of HCC1 into ECFP-C1 and EYFP-C1 (Clonetech). EcoR1 and BamH1 sites were introduced for the ligation of the other constructs. SF2/ASF EGFP-C1 and SF2/ASF AAA EGFP-C1 were a gift from Giuseppe Biamonti (Pavia) and were subsequently subcloned into ECFP-C1 and EYFP-C1. SRp53 was previously cloned into EGFP-C1 by Demian Cazalla, a former PhD student in the lab, and was subsequently subcloned into ECFP-C1 and EYFP-C1. U2AF35 and U2AF65 in ECFP-C1 and EYFP-C1 were a gift from Angus Lamond

(Dundee). All constructs generated in ECFP-C1 and EYFP-C1 vectors were subcloned into EGFP-C1 (Clonetech) and mCherry-C1 (Shaner et al., 2004).

Primer	Sequence
SRp20-F(EcoR1)	TCG**GAATTC**TCATCGTGATTCCTGTCCATTGGACT
SRp20-R(BamH1)	TCG**GGATCC**TTTCCTTTCATTTGACCTAGATCGA
SC35-F(EcoR1)	TCG**GAATTC**TAGCTACGGCCGCCCCCCTCCCGAT
SC35-R(BamH1)	TCG**GGATCC**AGAGGACACCGCTCCTTCCTCTTCAGGA
SF2/ASFΔRS-F(EcoR1)	TCG**GAATTC**TTCGGGAGGTGGTGTGATT
SF2/ASFΔRS-R(BamH1)	TCG**GGATCC**GCCATAGCTCGGGCTACG
Luc7A-F(EcoR1)	TCG**GAATTCT**ATTTCGGCCGCGCAGTTGTTGGAT
Luc7A-R(BamH1)	TCG**GGATCC**ATTGGACTGAGTGTCACCTTCAGA
U1-70K-F(EcoR1)	TCG**GAATTC**TACCCAGTTCCTGCCGCCCA
U1-70K-R(BamH1)	TCG**GGATCC**CTCCGGCGCAGCCTCCATCAA
T7-U170K-F(XbaI)	TCG**TCTAGA**ACCCAGTTCCTGCCGCCCA
T7-U170K-R(BamH1)	TCG**GGATCC**TCACTCCGGCGCAGCCTCCATCAA
HCC1.4-F(BglII)	TCG**AGATCT**GCAGACGATATTGATATTGAAGCA
HCC1.4-R(BamH1)	TCG**GGATCC**TCGTCTACTTGGAACCAGTAGCT

Table 2.1: Primers used for cloning the constructs used in this thesis. Restriction sites are shown in bold ,underlined.

2.2 Cell culture and transfections

HeLa and 293T HEK cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, and incubated at 37°C in the presence of 5% CO_2. HeLa and 293T HEK cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacture's instructions.

2.3 Cell fixation and immunofluorescence microscopy

HeLa cells grown on glass coverslips were fixed for 5 min in freshly prepared PBS/3.7% paraformaldehyde at RT. Permeabilisation was performed with PBS /1% triton x100 for 10 min at RT. After extensive washing, samples were blocked with 0.05% Tween20/PBS containing 1% goat serum (Sigma-Aldrich) for at least 30 min at RT, and then incubated for 1h with the primary anti-BrdU antibody (B2531; 1:500) or monoclonal SC35 (1:500). Secondary antibodies used were AMCA-conjugated goat anti–mouse (1:500; Jackson ImmunoResearch Laboratories) or TxRed anti-mouse (1:100) for 45 min incubation at RT. Then, coverslips were mounted in Vectashield medium (Vector Laboratories). The samples were observed on a Zeiss Axioskop microscope and the images were acquired with a Photometics CH250 cooled CCD camera using Digital Scientific Smartcapture extensions in software from IP Lab spectrum.

2.4 Fluorouracil (5-FU) incorporation assay

24 h after transfection, HeLa cells, either mock-treated or treated with DRB for the indicated length of time, were incubated with 2 mM 5-FU (F5130) for 30 min at 37°C. Subsequently, cells were fixed, permeabilized, and incubated with primary anti-BrdU antibody (B2531; 1:500). Immunofluorescence microscopy was performed as indicated (see section 2.3).

2.5 Western blot analysis

Samples were separated by SDS-PAGE and electroblotted Protan BA85 NitroCellulose (Schleicher and Schuell) in 25mM Tris-base, 40mM glycine and 20 % methanol in a Genie Blotter unit (Idea Scientific Company), at 12 volts for one hour. The membranes were blocked with 1:10 Western Blotting reagent (Roche) in TBST (20 mM Tris pH7.5, 137 mM NaCl and 0.1% Tween 20) for one hour at room temperature or overnight at 4°C. Incubations with primary and secondary antibodies were carried out for one hour at room temperature in TBST containing 1:20 Western Blotting reagent (Roche). Four washes with TBST were done after incubations with each antibody, and immunoreactive bands were detected with SuperSignal system (Pierce) according to the manufacturer's instructions. The following primary antibodies were used: mouse anti-GFP at 1:1000 (Roche) mouse anti-U1-70K at 1:1000 (Synaptic Sciences), rabbit anti-HCC1 at 1:5000 (Bethyl Laboratories), mouse anti-U2AF65 at 1:200 (a gift from Juan Valcarcel), mAb 96 at 1:500 (for detection of SF2/ASF), sheep anti-U2AF35 at 1:500. The appropriate secondary antibodies (horseradish peroxidase conjugated to IgG) were used at 1:10,000.

2.6 Immunoprecipitation

For Immunoprecipitation (IP) 293T cells that were transfected with a construct expressing the protein of interest or mock transfected were resuspended in 400 µl of lysis buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.3% NP40, 1mM PMSF) and incubated for 10 min at 4°C. The extract was

centrifuged at 12,000 x g for 20 min at 4°C, after which the pellet was discarded. The extract was incubated with the antibody of choice bound to 20 µL of protein A (Amersham) at 4°C for 2 h with continuous rotations. The IP reactions were then washed four times with lysis buffer. In some cases beads were treated with 50 µg/ml of RNase A/T1 cocktail (Ambion) for 10 min at 4°C. After RNase treatment, beads were resuspended in 30 µl of loading buffer (50 mM Tris [pH7.5], 10% glycerol, 0.05% SDS, 2.5% β-mercaptoethanol) and boiled for 3 min. For Western blot analysis of immunoprecipitated proteins, 10 µl of sample was used.

2.7 FRET Acceptor Photobleaching

HeLa cells grown on glass coverslips were cotransfected with an ECFP and EYFP construct of choice. Eight to 12 h post-transfection, cells were mounted in HEPES buffered Phenol red–free medium (Invitrogen Life Technologies) in a closed, heated chamber (Bachofer). Measurements were conducted on a DeltaVision Spectris Image Restoration Microscope (Applied Precision) fitted with a Quantifiable Laser Module (QLM) including a 20-mW 532-nm CW laser, suitable for photobleaching YFP without cobleaching CFP. Images were collected using an Olympus 60× NA 1.4 Plan-Apochromat lens, a Photometrics CoolSnap HQ cooled CCD camera and SoftWorx (Applied Precision) imaging software. The following specific CFP/YFP filter sets were used to resolve the ECFP and EYFP signals: excitation 436/10 nm and emission 480/40 nm for ECFP; excitation 525/20 nm and emission 580/70 nm for EYFP. The dichroics used were custom-built by Applied Precision and Chroma Technology. The set is modified from the normal CFP/YFP JP4 set such that the dichroic reflects and

the emission filter rejects light at 532 nm, allowing this wavelength to be used for selectively photobleaching YFP. After obtaining five pre-bleach images, a defined region of the cell nucleus was spot photobleached with a single 150-msec stationary pulse at 90% laser power. The first image was acquired 2 msec after the bleach event. For the first sec, images were acquired approximately every 200 msec; for the following 1.7 sec, every 335 msec; and then at 830-msec intervals in the following 5 sec, after which images were acquired every 1.6 sec for the remainder of the experiment. A total of 20 images were acquired after the bleach event. Images of donor (ECFP) and acceptor (EYFP) were taken in separate subsequent measurements, bleaching exactly the same spot before collecting post-bleach images. Obtained data were analyzed using the image analysis tools included in the SoftWorx software and the biostatistics program GraphPad Prism. In addition to the bleached region, a similar nonbleached nuclear region in the same cell was included in the data analysis as a control. A region of background fluorescence was defined outside the cell and subtracted from both the bleached and control regions. The data were normalised against the mean intensity of the whole image over time to account for any fluctuations and normal photobleaching that occur during image acquisition throughout the course of the experiment. FRET efficiency was calculated by the following formula:

$$\text{FRET Efficiency} = (I_{D(post)} - I_{D(Pre)} / I_{D(post)})$$

where $I_{D(pre)}$ and $I_{D(post)}$ are donor intensity before and after photobleaching, respectively. A FRET efficiency of greater than 5% is considered a significant protein-protein interaction based on the negative controls using YFP-NLS.

For inhibition of transcriptional activity, cells were treated for 2 h with 25 µg/mL DRB before carrying out FRET analyses by acceptor photobleaching.

2.8 Fluorescence lifetime measurements by time-correlated single-photon counting (TCSPC) for FRET experiments

Cells were prepared as described for acceptor photobleaching assays except EGFP and mCherry fluophores were used. Fluorescence lifetime Imaging Microscopy (FLIM) was performed using an inverted laser scanning multiphoton microscope (Nikon TE2000/Bio-Rad Radiance 2100MP) with a 63x oil immersion (NA 1.4). Two-photon excitation was achieved using a Chameleon^{TM} Verdi pumped ultrafast tunable (720-930 nm) laser (Coherent Inc.), to pump a mode-locked frequency-doubled Ti:Sapphire laser (Coherent) that provided sub-200 femtosecond pulses at a 90 Mhz repetition rate with an output power of approximately 1.4W at the peak of the tuning curve (800 nm). Enhanced detection of the scattered component of the emitted (fluorescence) photons was afforded by the use of fast single-photon response (Hammamatsu 5783P) direct detectors. The fluorescence lifetime imaging capability was provided by time-correlated single photon counting electronics (SPC-830; Becker & Hickl GmbH). The optimal two-photon excitation wavelength to excite the donor (EGFP) was determined to be 890 nm. Fluorescence emission of EGFP- fusion proteins was collected using a bandpass filter (525 ± 25 nm) to limit detection to only the donor fluorophore (EGFP) and prevent contamination from the acceptor (mCherry) emission. Laser power was adjusted to give a mean photon count rate of the order 10^4 to 10^5 photons/s, and fluorescence lifetime images were acquired over 120 sec. Fluorescence lifetimes were calculated for all pixels in the field of view (256 x 256 pixels) using SPCImage software. A bi-exponential fluorescence decay model is applied to the data to determine the fluorescence lifetime of non-interacting and interacting subpopulations.

Measurements of FRET based on analysis of the fluorescence lifetime of the donor can distinguish between FRET efficiency (i.e., coupling efficiency) and an increase in FRET population (concentration of FRET species). The assumption that non-interacting and interacting fractions are present allows us to determine both the efficiency of interaction and the fraction of interacting proteins. Such a FLIM analysis can be enhanced by extending from a pixel to pixel analysis to a global analysis (i.e., assumption of globally invariant fluorescence lifetime components and calculation over all pixels throughout the measured cell) (Verveer et al., 2000a). By fixing the non-interacting species lifetime using data obtained from control experiments (in the absence of (FRET)), more accurate estimations of the remaining free parameters may be made. Furthermore, by assuming invariance in the efficiency of interaction between pixels throughout the measured cell one can determine the % of interacting species (a1) by summation of data throughout the cell and thereby fix both lifetime parameters. Mean FRET efficiency images were calculated such that the FRET efficiency, $E_{FRET} = 1 - \tau_{DA}/\tau_D$ where τ_{DA} is the mean fluorescence lifetime of the donor in the presence of the acceptor and τ_D is the mean fluorescence lifetime of the donor in the absence of acceptor for all the cells imaged. In order to calculate the mean FRET efficiency the non-interacting species lifetime was fixed using data obtained from control experiments. A FRET efficiency of greater than 5% is considered a significant protein-protein interaction based on the FRET efficiencies measured using mCherry-C1 as a negative control.

Results

3.1 Choice of fluorphores for FRET analysis

This thesis characterises interactions between splicing factors in living human cells using two techniques: FRET acceptor photobleaching and Fluorescent Lifetime Imaging Microscopy (FLIM). For FRET acceptor photobleaching analyses enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) fusions of the splicing factor of interest were generated. For FLIM analyses, enhanced green fluorescent protein (EGFP) and mCherry fusion (Shaner et al., 2004) of splicing factors were constructed.

GFP is a 27-kDa monomer consisting of 238 amino acids (Prasher et al., 1992). The peak emission for GFP is 509 nm and the peak excitation is 488 nm. The extensive mutagenesis of GFP has yielded fluorescent protein variants with different excitation and emission properties, providing several protein tags with suitable spectral overlap for FRET studies (Heim and Tsien, 1996; Tsien, 1998; Zhang et al., 2002). Early FRET studies used blue fluorescent protein (BFP) as a donor fluorophore and either YFP or GFP as an acceptor fluorophore (Mitra et al., 1996; Day et al., 2001b). However, the quantum yield of BFP is very low, and it is very susceptible to photobleaching (Rizzuto et al., 1996). Some of the intrinsic problems of using BFP were overcome when CFP was identified (Heim and Tsien, 1996). The peak emission for CFP is 476 nm and the peak excitation is 434 nm. The YFP variant (Heim et al., 1995; Tsien, 1998) is the most red shifted of the mutant variants of GFP yet generated, with a peak emission at 527 nm and peak excitation at 514 nm. CFP and YFP have become a popular FRET pair due to the good spectral overlap, with CFP

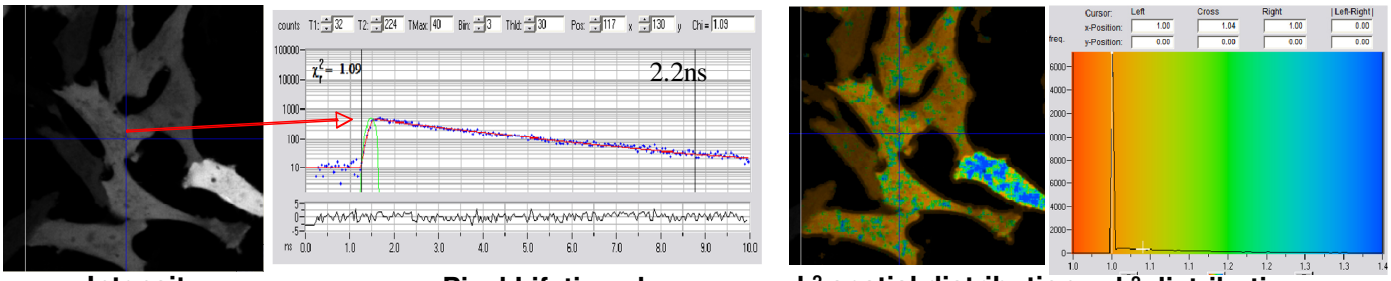
acting as a donor and YFP an acceptor (Miyawaki et al., 1997). YFP has a high quantum yield and its susceptibility to photobleaching is an advantage in FRET acceptor photobleaching studies (Tsien, 1998).

Red fluorescent protein (RFP) was cloned from the sea anemone Discosoma striata (Matz et al., 1999). RFP is a 28-kDa protein that shares about 25% sequence identity with GFP. RFP is a very slow maturing protein that has a strong tendency to form tetramers *in vivo* and *in vitro* (Baird et al., 2000). The first true monomer was mRFP1, derived from the Dicosoma sp. fluorescent protein DsRed (Bevis and Glick, 2002; Campbell et al., 2002). Subsequent mutagenesis has yielded monomers (Shaner et al., 2004), such as mCherry (Excitation maximum 587 nm, Emission maximum 610 nm) that mature more completely, are more tolerant of N-terminal fusions and are more photostable than mRFP1. The spectral overlap between GFP and mCherry make them suitable for FRET analysis with GFP acting as the donor and mCherry the acceptor (Tramier et al., 2006).

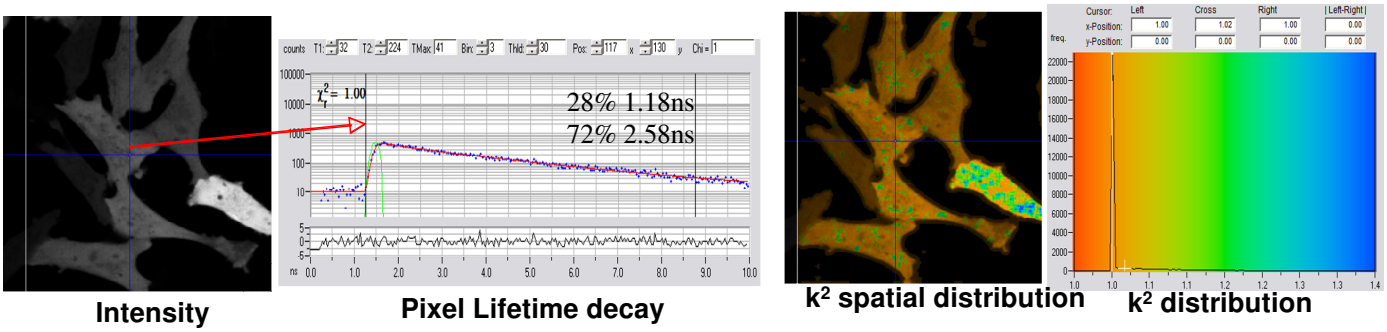
3.2 Development of FLIM methods

During the early stages of this thesis a number of Time-correlated single-photon counting (TCSPC) FLIM experiments were carried out using ECFP and EYFP as the donor and acceptor pair. However, there are numerous problems with analysing the data from FLIM experiments performed using ECFP and EYFP. Previously it has been shown that CFP alone may exhibit bi-exponential decay kinetics when observed in a cellular environment (Tramier et al., 2002). Models predict that there is a short lifetime associated with ECFP of 1.18ns and a longer lifetime of 2.58ns.

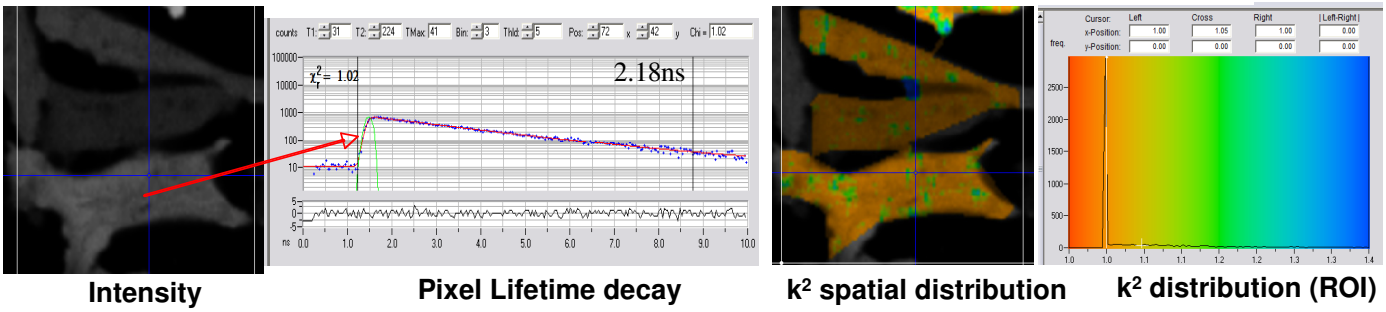
A **Experimental Fitting Data: Single-exponential decay model**



Experimental Fitting Data: Double-exponential decay model



B **Experimental Fitting Data: Single-exponential decay model**



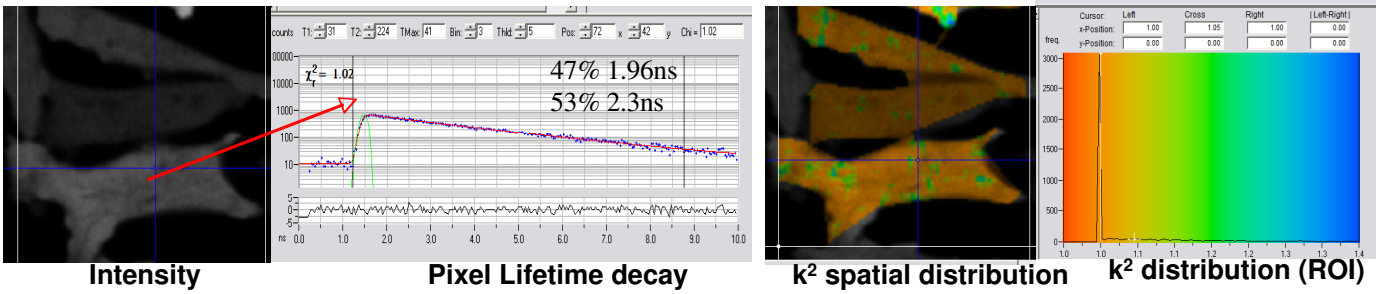


Figure 3.1: ECFP has two lifetimes and EGFP only one (A) Lifetime decay graphs and K^2 distributions are shown for ECFP fitted to a single or a double exponential decay model. **(B)** Lifetime decay graphs and K^2 distributions are shown for EGFP fitted to a single or a double exponential decay model.

Figure 3.1 shows the pixels lifetime decay curves and K^2 distribution for ECFP and EGFP. The lifetime decays can be fitted to either single or double exponential decay models which assume there are either one or two lifetimes present respectively. The K^2 distributions are a measure of how well the data fit the model. The closer the value is to one the better the fit. Importantly it was observed that when a double exponential decay model is applied for the fluorescent decay of ECFP the K^2 is reduced from 1.09 in the single exponential decay model to 1.00. However, when a double-exponential decay model is applied for the fluorescent decay of EGFP no improvement in the K^2 is observed. Therefore, while the double-exponential decay model can determine two lifetimes for EGFP this is less accurate than assuming that there is one lifetime present. In summary, by fitting the data to the model to give the best K^2 value, there are two fluorescent lifetimes associated with ECFP and only one with EGFP.

The double-exponential decay model shows that there is a short lifetime associated with ECFP of 1.18ns and a loger lifetime of 2.58ns. This leads to complications when analysing the data from FRET experiments. In experimental FRET situations free donors co-exists with donors bound to acceptors. This means that the fluorescent decay curves for FRET experiments will contain two components. Therefore the mean fluorescent lifetime can be represented by the equation: $tm = a_{1FRET}t_{1FRET} + a_2t_2$, where t_1 is the short lifetime of the binding donor fraction a_1, and t_2 is the long lifetime of the free donor fraction a_2. However, it has been demonstrated that ECFP has a double exponential decay therefore the short fluorescent lifetime observed in experimental situation is due to both FRET and the short lifetime of ECFP making quantification difficult.

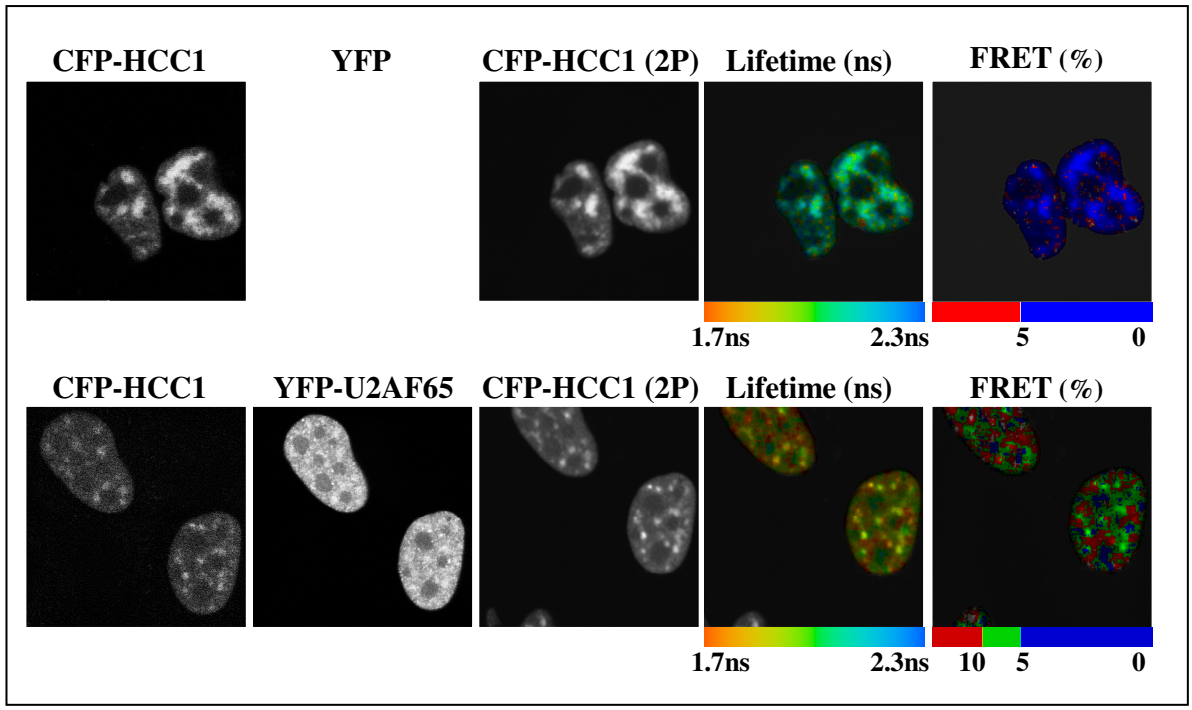


Figure 3.2: FRET between ECFP-HCC1 and EYFP-U2AF65 detected by FLIM. HeLa cells were transfected with ECFP-HCC1 and cotransfected with EYFP-U2AF65. Confocal image of transfected cells and FLIM image of same cells, in which mean fluorescent lifetime is shown in false colour. The colour scale with the respective lifetimes (in picoseconds) is indicated.

Figure 3.2 shows that FRET can be detected between ECFP-HCC1 and EYFP-U2AF65 in FLIM experiments. Interestingly, the same pattern of interactions were observed using either ECFP and EYFP FRET pairs or EGFP and mCherry FRET pairs (see section 3.16). However, due to the problems described above with the bi-exponential decay of ECFP, FRET between ECFP and EYFP is considered more qualitative than quantitative. Further doubts about the accuracy of FLIM experiments using ECFP and EYFP have been cast due to the photosensitivity properties of ECFP under the mercury lamp which can influence fluorescent lifetimes in unpredictable ways (Tramier et al., 2006). Due to these limitations of using ECFP and EYFP, I considered EGFP and mCherry to be a more suitable FRET pair for the FLIM experiments described in this thesis.

3.3 Fluorescently tagged proteins colocalise with endogenous SC35 in speckles

Some concerns have previously been expressed that the fusion of the 27-kDa GFP tag to a protein can affect its function within the cell. However, there is mounting evidence that fluorescently tagged proteins are functional such as the case of fluorescently tagged snRNP proteins that have been shown to assemble into snRNPs (Stanek and Neugebauer, 2004). Furthermore, GFP-SF2/ASF has been shown to be functional in *in vivo* splicing assays (Misteli et al., 1997). To determine whether the presence of the fluorescent protein tag affected the subcellular distribution of the splicing factors cloned for FRET analysis indirect immunofluorescence was carried out in HeLa cells. Cells were transiently transfected with the N-terminal GFP tagged protein, fixed and stained with an antibody specific for SC35, a marker of speckles. The merged images in Figure 3.1 demonstrate that all the EGFP-tagged proteins colocalise with SC35 and show a typical speckled pattern. The other fluorescently tagged proteins (ECFP, EYFP, mCherry) showed a similar pattern upon transient transfection (data not shown). This led to the conclusion that the presence of the fluorescent protein tag does not affect the subcellular distribution of any of the proteins analysed (Misteli et al., 1997).

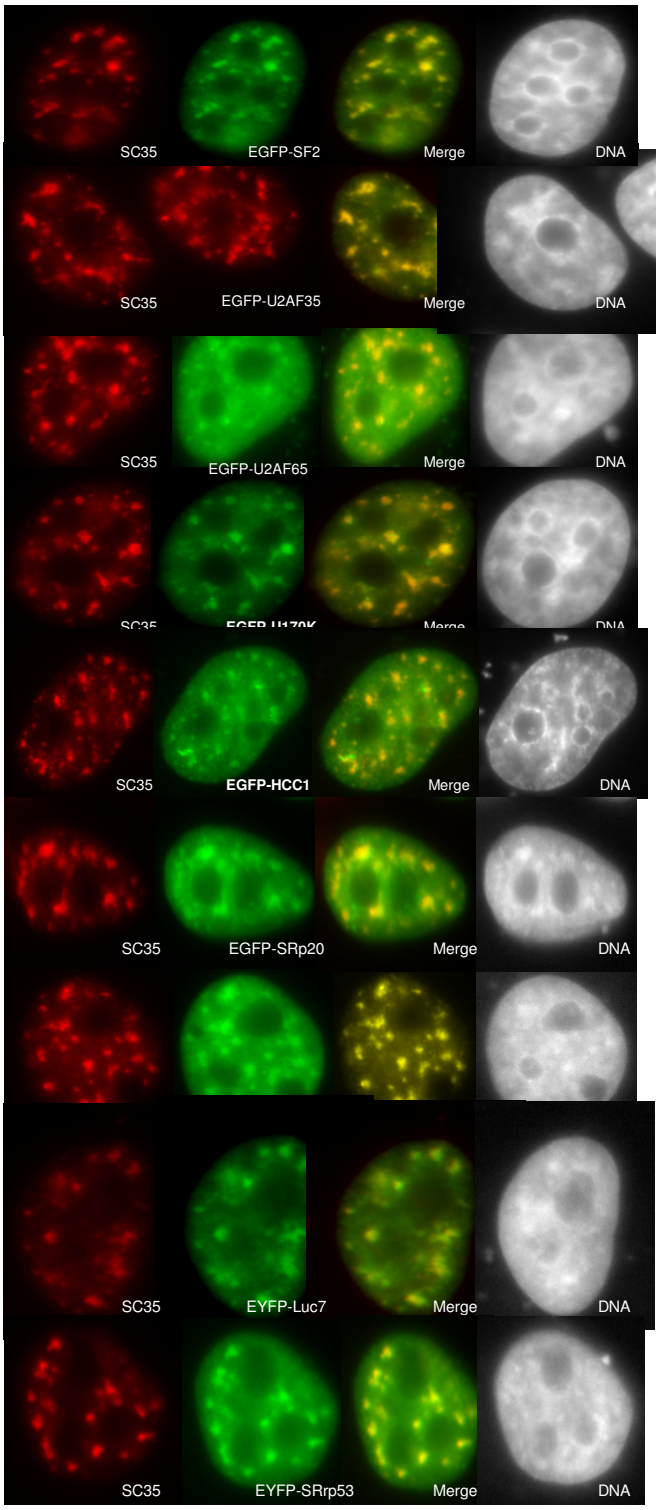


Figure 3.3: EGFP-tagged proteins colocalise in nuclear speckles with endogenous SC35. HeLa cells transiently expressing EGFP tagged splicing factors were fixed 12 hours post transfection. Endogenous SC35 was detected by indirect immunoflouscence with an anti-SC35 monoclonal antibody.

A further aim of this thesis was to analyse whether mutations in SF2/ASF affect its protein interactions in living cells. In order to determine this, mutant versions of SF2/ASF were constructed that either had a point mutation in the second RRM (SF2/ASF AAA) or lacked the RS domain (SF2/ASFΔRS) (See section 3.14 for details). Previous indirect immunofluorescence with T7 epitope-tagged SF2/ASFΔRS (T7-SF2/ASFΔRS) has demonstrated that deletion of the RS domain of SF2/ASF results in a weak cytoplasmic accumulation although it is still recruited to the speckles (Caceres et al., 1997). It has also been reported that point mutations in RRM2 of SF2/ASF do not affect its subcellular localisation (Chiodi et al., 2004). Figure 3.2 demonstrates that fluorescently tagged SF2/ASFΔRS and SF2/ASFAAA localised as would be expected based on previous studies when transiently transfected into HeLa cells. EGFP-SF2/ASFΔRS showed a weak cytoplasmic staining in addition to colocalising with SC35 in speckles whereas the mutation in EGFP-SF2/ASFAAA did not affect its localisation.

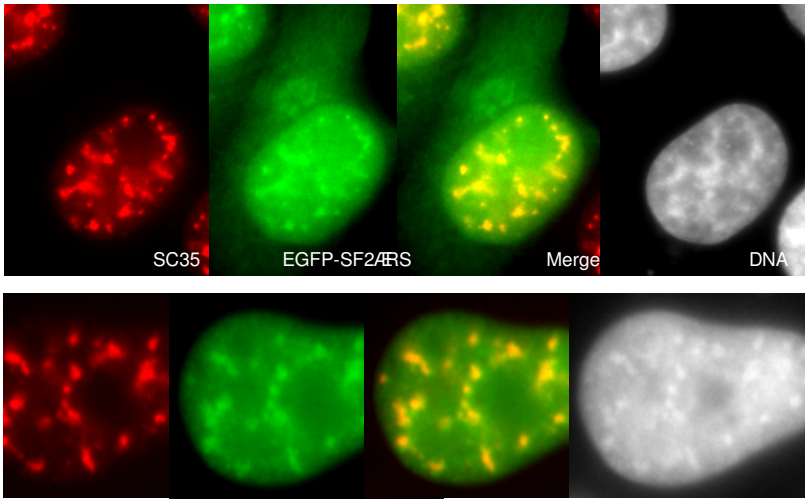


Figure 3.4: EGFP-tagged SF2/ASFΔRS and SF2/ASF AAA show the expected localisation patterns. HeLa cells transiently expressing EGFP-tagged SF2/ASF mutants were fixed 12 hours post transfection. Endogenous SC35 was detected by indirect immunoflouscence with an aniti-SC35 monoclonal antibody.

3.4 Expression levels of full-length fluorescent proteins

To determine the expression level of the fluorescently tagged proteins and to confirm that they are expressed as full-length proteins western blot analysis was carried out. 293T cells were transiently transfected with the EGFP-tagged constructs and 12 hours post transfection the lysates were analysed by SDS-PAGE. Where available western blots were carried out with antibodies raised against the endogenous protein, when this was not available anti-GFP was used. Figure 3.3 shows that the antibodies recognised the endogenous protein and the GFP-tagged protein shifted by 27 kDa and no degradation bands were visible. Therefore, it was concluded that all the fluorescently tagged proteins are full length. Each band ran at the expected molecular weight but due to different constructs being run on different gels a comparison been individual gels cannot be made. Figure 3.3 also shows that the expression levels of the tagged proteins are similar to that of the endogenous protein when averaged out over a population of 293T cells.

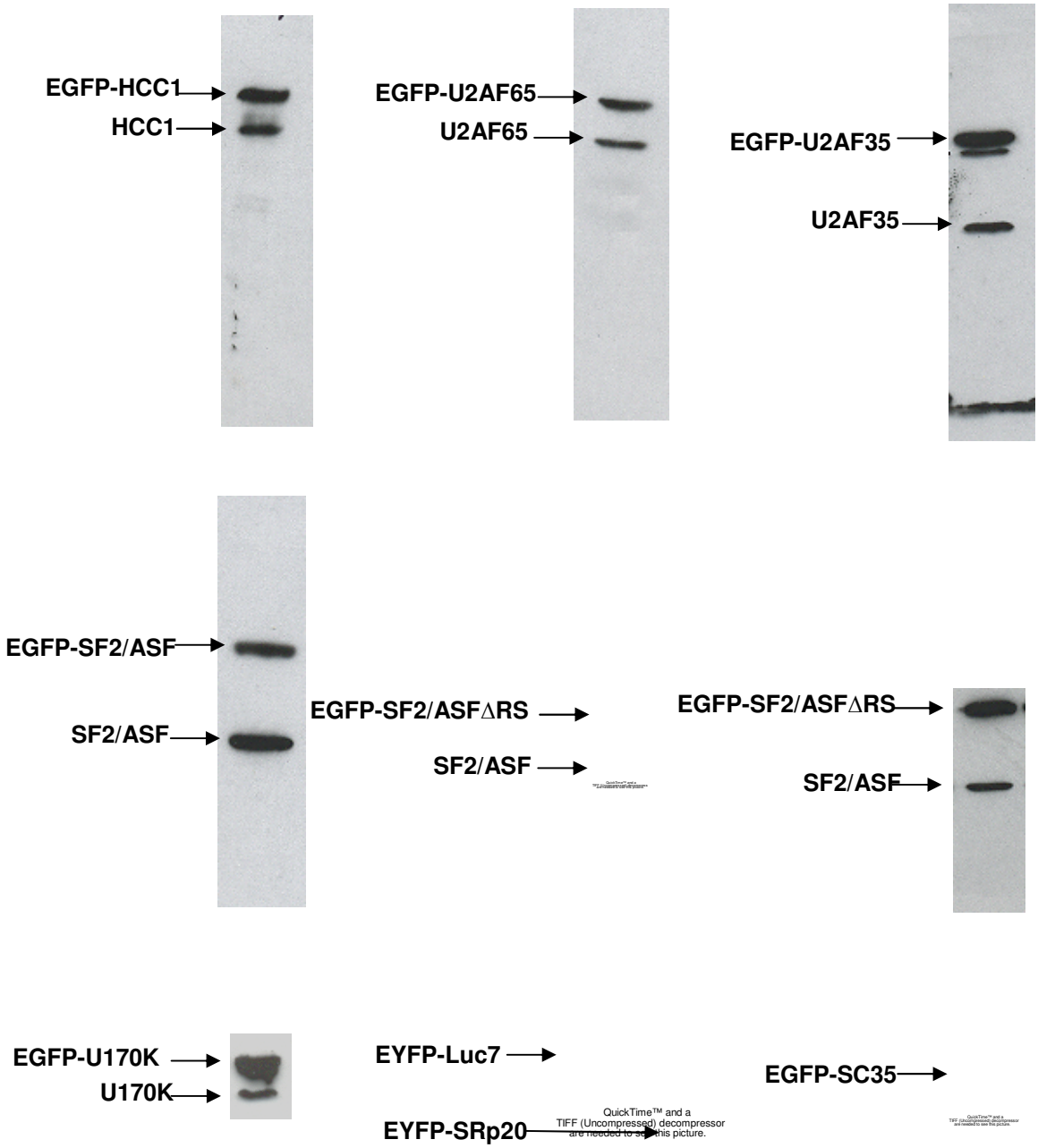


Figure 3.5: Characterisation of the expression levels of transiently transfected flourescently tagged proteins in 293T cells. 12 hours post transfection total lysates were prepared from 293T cells. Extracts were separated on an SDS 12% polyacrylamide gel and analysed by Western blotting with the appropriate endogenous antibody or with anti-GFP for the detection of EGFP-SC35, EYFP-SRp20 and EYFP-Luc7.

3.5 FRET acceptor photobleaching demonstrates the interaction of SF2/ASF with U170K in live cells

U1 snRNP is the earliest snRNP to assemble on pre-mRNAs (Mount et al., 1983; Ruby and Abelson, 1988) defining the 5' splice site via RNA-RNA and RNA-protein interactions (Zhuang and Weiner, 1986; Zhuang et al., 1987). It has been demonstrated that SR proteins collaborate with U1 snRNP in 5' splice site recognition: purified U1 snRNP and SF2/ASF form a ternary complex with pre-mRNA, which is dependent on a functional 5' splice site (Kohtz et al., 1994). Additionally, SF2/ASF has been shown to enhance the affinity of U1 snRNP for 5'splice sites (Eperon et al., 2000). This effect is mediated directly by an interaction between SR proteins and U170K (Wu and Maniatis, 1993). Previously it has been demonstrated that recombinant SC35 and SF2/ASF can interact with U170K when immobilised on a nitrocellulose filter or in solution. This interaction has also been demonstrated in yeast two-hybrid assays (Wu and Maniatis, 1993).

The FRET acceptor photobleaching assay was utilised to study this interaction in living HeLa cells. This allowed the interactions to be studied while preserving the natural salt concentrations and dynamic structure of the nucleus.

In these experiments ECFP and EYFP serve as the donor and acceptor pair for FRET, respectively. If the donor (ECFP) and acceptor (EYFP) are in close proximity (<10 nm) and in the appropriate relative orientation to each other, excitation of the donor molecule leads to transfer of energy to the acceptor. This energy transfer results in a decrease in fluorescence emission from the donor and an increase in emission of the acceptor. To measure FRET by acceptor photobleaching the donor fluorescent

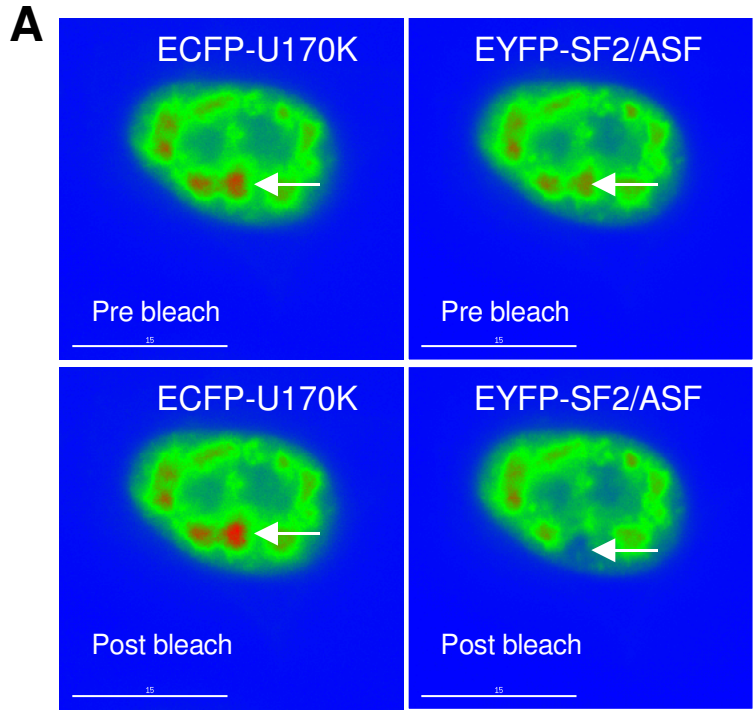
emission is measured, comparing the quenched with the unquenched donor emission after specific photobleaching of the acceptor fluorophore. In the case of FRET occurring, photobleaching of the acceptor results in a transient enhancement in donor emission. This dequenching effect indicates an abolishment of FRET due to photobleaching of the acceptor fluorophore, and thus confirms that the two proteins interact directly *in vivo*.

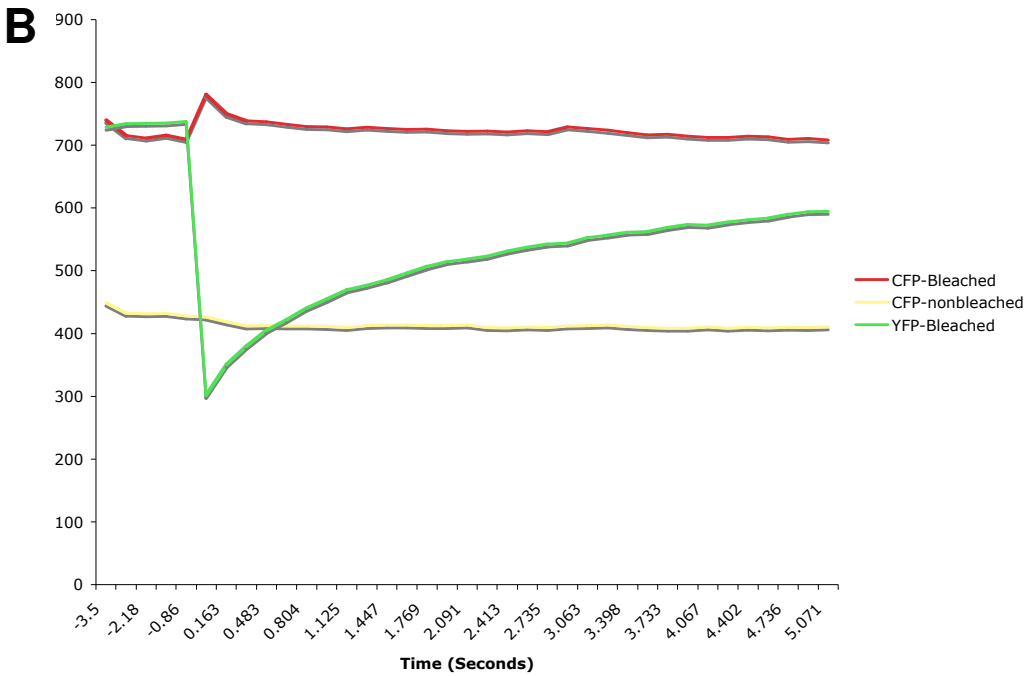
ECFP-U170K and EYFP-SF2/ASF were transiently transfected into HeLa cells. Images were acquired before and after photobleaching the acceptor with a single 150-msec stationary laser pulse (Figure 3.4(A)). Speckles were chosen as the region of interest in all the photobleaching experiments described. The post-bleach images, collected 2 msec after the laser pulse, clearly show the dequenching effect of the donor as a result of bleaching of the acceptor. Due to the rapid diffusion of unbleached acceptor into the bleached area dequenching of the donor was detectable only for a short time period. Dequenching was only observed in the area that had been photobleached. Figure 3.4(B) shows donor (bleached and unbleached) and acceptor mean signal intensities plotted over time.

In order to calculate a FRET efficiency for this interaction the following formula was used:

$$\text{FRET Efficiency} = (I_{D(post)} - I_{D(Pre)} / I_{D(post)})$$

Where $I_{D(pre)}$ and $I_{D(post)}$ are mean donor intensity before and after photobleaching, respectively. In order to account for variations in mean signal intensity 5 pre-bleached images were taken and the average donor intensity was calculated. A FRET efficiency of greater than 5 % is considered significant. Figure 3.4(C) shows that significant FRET is observed between ECFP-U170K and EYFP-SF2/ASF.


A
ECFP-U170K
EYFP-SF2/ASF
Pre bleach
Pre bleach
ECFP-U170K
EYFP-SF2/ASF
Post bleach
Post bleach


B
900
800
700
600
500
400
300
200
100
0
-3.5
-2.18
-0.86
0.163
0.483
0.804
1.125
1.447
1.769
2.091
2.413
2.735
3.063
3.398
3.733
4.067
4.402
4.736
5.071
Time (Seconds)
CFP-Bleached
CFP-nonbleached
YFP-Bleached

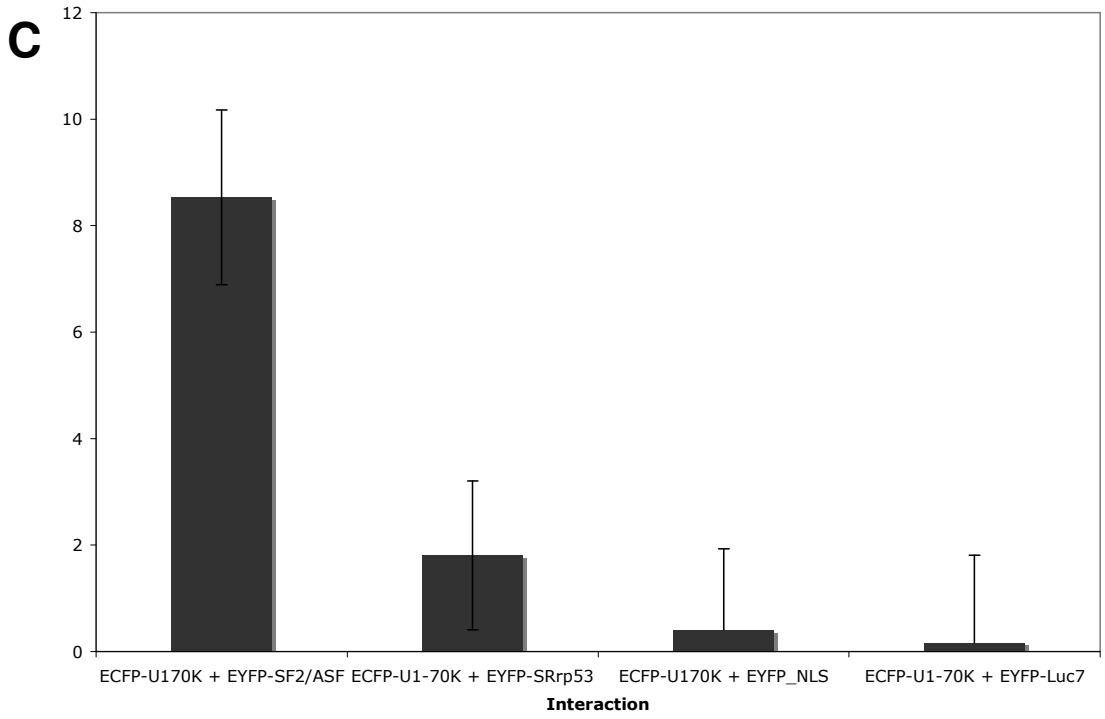


Figure 3.6: (A) *In vivo* detection of protein-protein interactions between ECFP-U170K and EYFP-SF2/ASF by FRET acceptor photobleaching microscopy. Live HeLa cells transiently coexpressing ECFP-U170K and EYFP-SF2/ASF were analysed on a wide-field fluorescent microscope equipped with a quantifiable laser module as described in Materials and Methods. Images were acquired before and after phobleaching with a single 150-msec stationary laser pulse. A nonbleached region similar to the bleached region (arrow) was included in the data analysis for comparison**. (B) Donor and acceptor mean signal intensities monitored in the bleached and nonbleached regions were plotted over time. (C) FRET efficiencies for the interaction between ECFP-U170K and EYFP-SF2/ASF.** Plot of FRET efficiencies (average for 8 to 27 cells) between CFP + YFP pairs measured by FRET acceptor photobleaching. FRET efficiency was calculated from CFP fluorescence before and after bleaching: $FRET_{efficiency}$ [%] = $100((CFP_{after} - CFP_{before}) / CFP_{after})$ A FRET efficiency of greater than 5% is taken as significant. Error bars show standard deviations.

It was important to demonstrate that the fluorescently tagged proteins show specificity for their interaction partners. Figure 3.4(C) shows that ECFP-U170K does not interact with an EYFP- tagged version of the second step splicing factor SRrp53 as previously reported by yeast two-hybrid analysis (Cazalla et al., 2005). In addition significant FRET was not observed between ECFP-U170K and an EYFP-tagged nuclear localisation signal (EYFP-NLS). Luc7p has previously been shown to be a component of the U1 snRNP with a role in 5' splice site recognition in yeast (Fortes et al., 1999). To determine if human Luc7A plays a similar role in mammalian cells I tested whether it interacts with U170K. Significant FRET was not observed for this interaction however, FRET cannot confirm a negative interaction and it could simply be that the fluorescent tags are not in a favorable orientation for energy transfer to occur. In conclusion I have employed FRET acceptor photobleaching to demonstrate the interaction between U170K and SF2/ASF that had previously been characterized by *in vitro* techniques occurs in living HeLa cells.

3.6 The interaction between U170K and SF2/ASF is not exclusively co-transcriptional

Splicing frequently occurs co-transcriptionally and it has been demonstrated that coupling transcription and splicing increases the fidelity and efficiency of splicing (reviewed by(Neugebauer, 2002)). In addition, the rate of pol II elongation has been shown to affect spliceosome assembly (Listerman et al., 2006) and alternative splicing (de la Mata et al., 2003).

The adenosine analogue 5,6-dichloro-1-b-d-ribofuranosylbenzimidazole (DRB) interrupts the elongation step of pol II transcription by promoting premature termination (Tamm et al., 1976; Fraser et al., 1978; Laub et al., 1980; Chodosh et al., 1989). Phosphorylation of the pol II C-terminal domain (CTD) has been suggested to accompany the transition from initiation to elongation steps of transcription. The blocking of transcription mediated by DRB may result from its ability to inhibit one or more protein kinases that phosphorylate the CTD of pol II (Dubois et al., 1994; Yankulov et al., 1995; Zandomeni et al., 1986). The positive transcription-elongation factor b (P-TEFb) has been implicated in mediating the inhibitory effects of DRB on transcriptional elongation (Marshall and Price, 1992).

To determine whether the interaction between U170K and SF2/ASF is dependent on splicing activity, live HeLa cells co-expressing ECFP-U170K and EYFP-SF2/ASF were treated with DRB to inhibit transcription, and hence splicing activity, before analysing the protein-protein interactions by acceptor photobleaching. DRB treatment resulted in a nuclear localisation pattern typical for splicing factors in transcriptional inhibited cells; i.e. the speckles become enlarged and rounded. As in transcriptionally active cells, photobleaching the acceptor led to a transient

enhancement in donor emission (Figure 3.5(A)). The FRET efficiency for the interaction between ECFP-U170K and EYFP-SF2/ASF is not significantly affected by treatment with DRB (Figure 3.6) as confirmed by a two-tailed homoscedastic t-test. To confirm that DRB treatment was having the predicted effects on transcription I carried out fluorouracil (5-Fu) labeling to visualise ongoing transcription before and after treatment with DRB. Figure 3.7 clearly shows that DRB treatment results in a reduction in the levels of nascent transcripts and the speckles become enlarged and rounded. Consistent with previous reports the effects of DRB were shown to be reversible by 5-Fu labeling in cells that had been incubated in normal media for 1 hour after DRB treatment. A recovery in the level of transcription can be seen and the speckles revert back to their normal morphology. In conclusion this data shows that U170K and SF2/ASF interaction still exist when transcription and therefore splicing is inhibited.

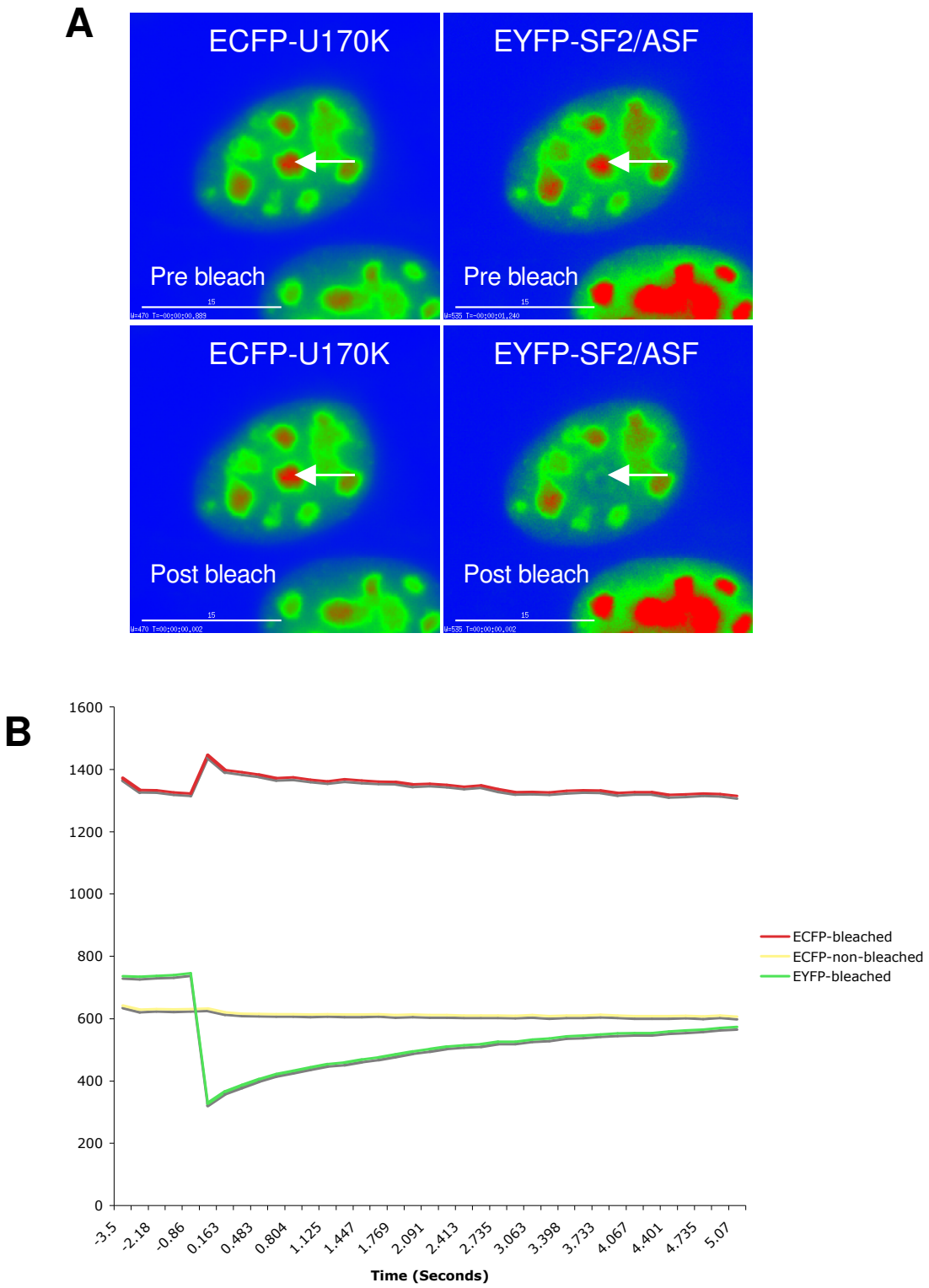


Figure 3.7: *In vivo* detection of protein-protein interactions between ECFP-U170K and EYFP-SF2/ASF by FRET acceptor photobleaching microscopy in the presence of DRB. Experiments were performed exactly as described for figure 3.4 except cells were treated with 25 µg/ml of DRB for 2 hours before images were taken. **(B) Donor and acceptor mean signal intensities monitored in the bleached and nonbleached regions of cells treated with DRB were plotted over time.**

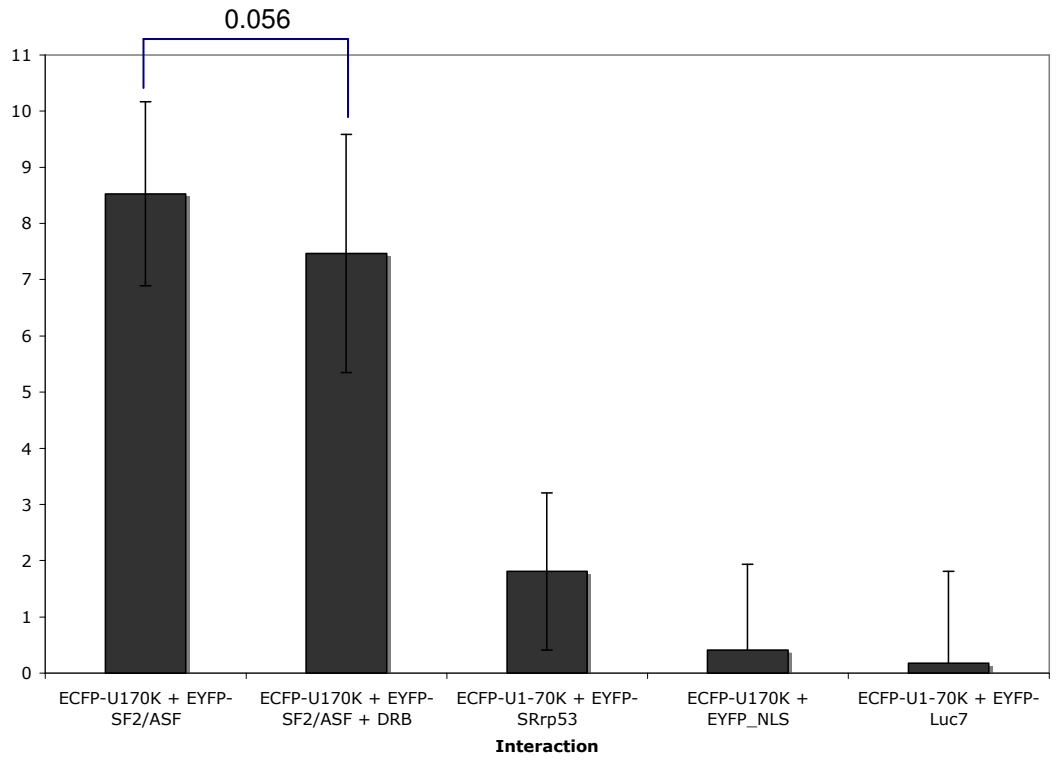
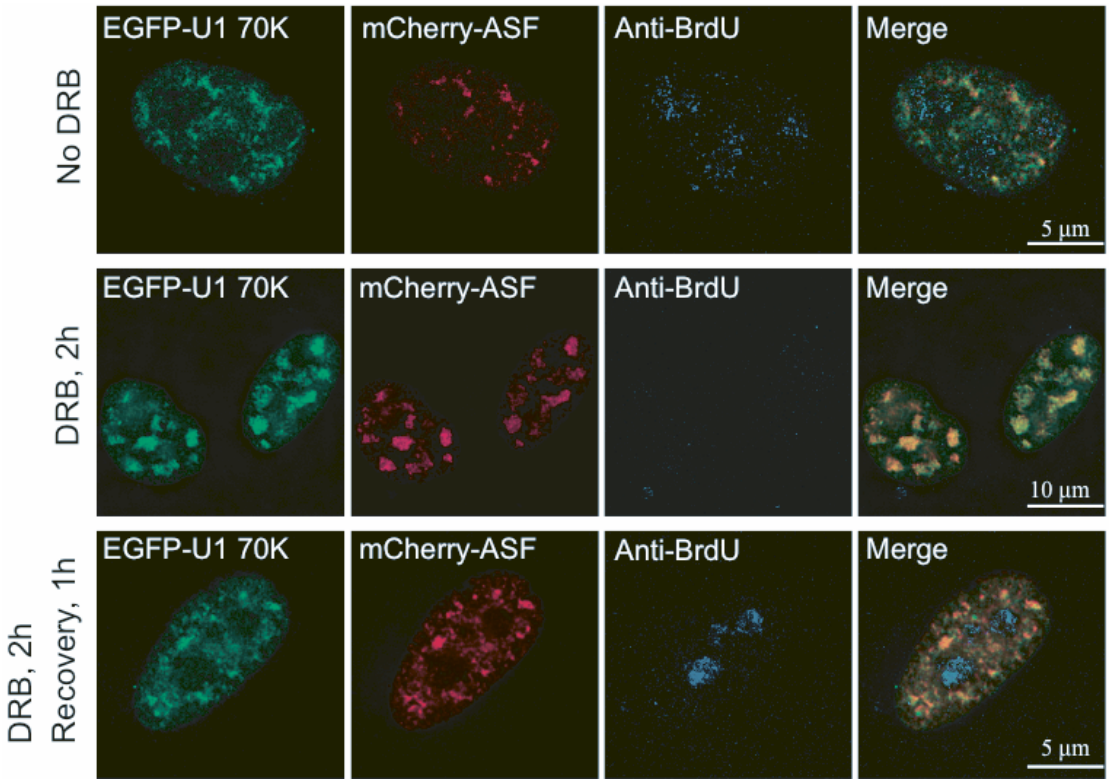


Figure 3.8: FRET efficiencies for the interaction between ECFP-U170K and EYFP-SF2/ASF in the presence and absence of DRB. Plot of FRET efficiencies (average for 8 to 27 cells) between CFP + YFP pairs measured by FRET acceptor photobleaching before and after treatment with 25 μg/ml of DRB for 2 hours. FRET efficiency was calculated from CFP fluorescence before and after bleaching: $FRET_{efficiency}$ [%] = 100((CFP_{after} − CFP_{before}) / CFP_{after}) A FRET efficiency of greater than 5% is taken as significant. Error bars show standard deviations.



Images were taken by David Lleres (Dundee)

Figure 3.9: DRB treatment inhibits transcription. HeLa cells were pulse labeled for 30 minutes with 5-FU and immunofluorescence was carried out with anti-BrdU to visualise nascent transcripts. DRB treatment was carried out for two hours with 25 µg/ml of DRB.

3.7 Mapping the interaction between U170K and SF2/ASF using FLIM microscopy

FRET acceptor photobleaching only gives a FRET efficiency for the specific area that is being photobleached within the cell. In order to precisely map the localisation of the U170K-SF2/ASF interaction within the nucleus of live HeLa cells I utilised FLIM microscopy.

In these experiments EGFP and mCherry serve as the donor and acceptor FRET pair. FLIM relies on the fact that in the absence of FRET EGFP has a stable fluorescent lifetime of approximately 2.3 ns in the cellular environment providing a standard reference point. As demonstrated with the acceptor photobleaching technique, FRET is a very powerful fluorescent quencher. Thus, a decrease in the fluorescent lifetime of the donor can be used to measure a FRET interaction with the acceptor flurophore.

FLIM was performed by the Time-correlated single-photon counting (TCSPC). This technique gives the picosecond time-resolved fluorescent decay directly for each pixel within the cell by by statistical analysis of the arrival times of photons with respect to the excitation pulse (O'Connor and Phillips, 1984).

The fluorescent lifetime of EGFP-U170K in transiently transfected live HeLa cells was measured either in the presence of mCherry-C1 (negative control) or mCherry-SF2/ASF. The donor decay curves were fitted to a double exponential decay model, providing information on the donor lifetimes of both the interacting (t1) and non-interacting populations (t2) within a cell. Figure 3.8(A) demonstrates that cotransfection of EGFP-U170K and mCherry-SF2/ASF results in a reduction of the

mean donor lifetime indicative of FRET. The images have been false coloured to show the mean fluorescent lifetime for each pixel within the cell.

In order to calculate the mean FRET efficiency for the interaction of EGFP-U170K with mCherry-SF2/ASF the following equation was used:

$$E_{FRET} = 1 - \tau_{DA}/\tau_D$$

Where τ_{DA} is the mean fluorescence lifetime of the donor in the presence of the acceptor and τ_D is the mean fluorescence lifetime of the donor in the presence of mCherry-C1 for all the cells imaged. A FRET efficiency of greater than 5% is considered significant. Figure 3.9 shows that significant FRET is observed between EGFP-U170K and mCherry-SF2/ASF. In figure 3.8(A) the images are false coloured in either continuous or discrete colour to show FRET efficiency and as expected this correlates with the changes in mean donor lifetime. It can clearly be seen that FRET between EGFP-U170K and mCherry-SF2/ASF occurs to a greater extent in the speckles compared to the nucleoplasm.

The total decrease in donor fluorescent lifetime depends on both the distance between the donor and acceptor and the fraction of the interacting donor molecules (Hoppe et al., 2002). The distance between the donor and acceptor (r) can be calculated from the equation:

$$r = R_0 ((1 - E_{FRET}) - 1)^{1/6}$$

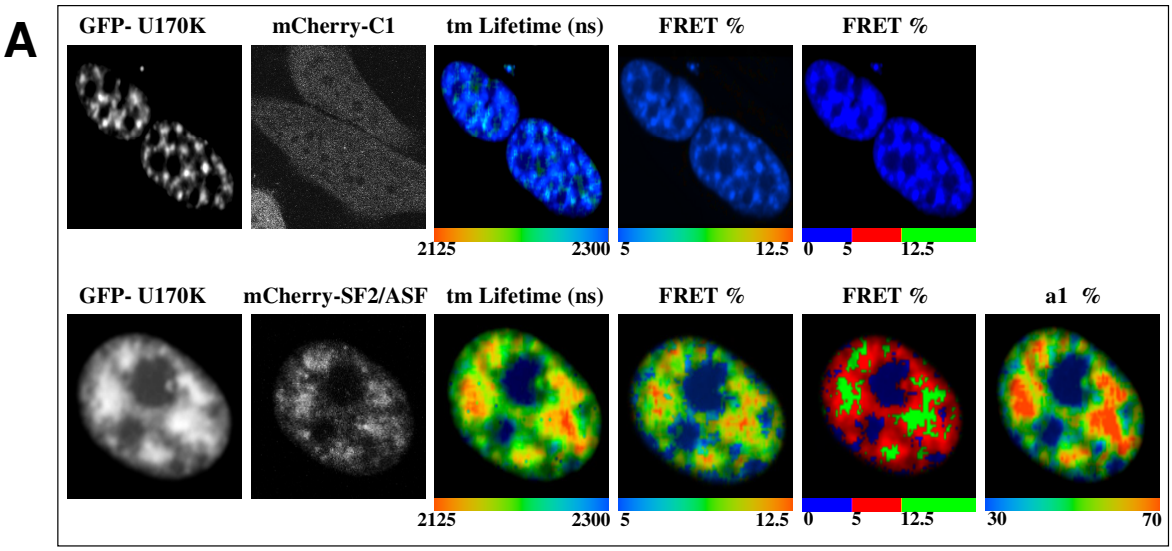
Where E is the energy transfer efficiency and R_0 is the Forster distance – that is, the distance between the donor and acceptor at which half the excitation energy of the donor is transferred to the acceptor.

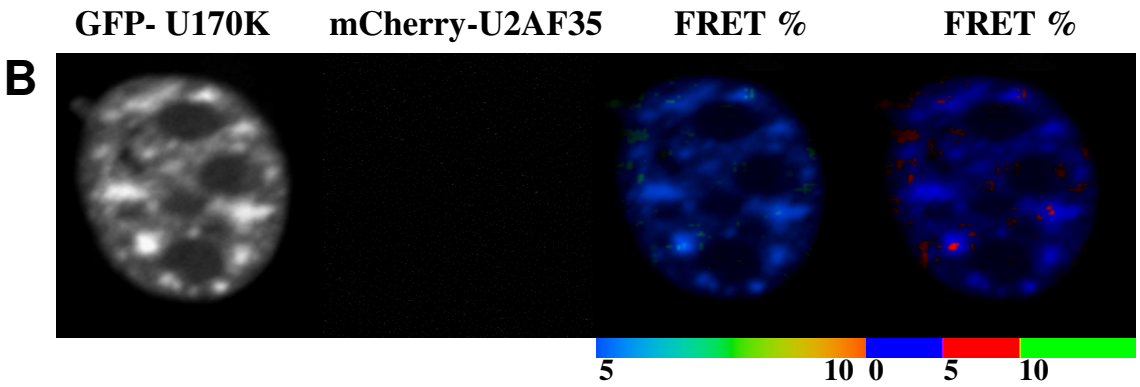
The fraction of interacting donor molecules can be calculated from the equation:

$$\text{Donor decay: } f(t) = a \times e^{-t/\tau_{FRET}} + b \times e^{-t/\tau_0}$$

Where $e^{-t/\tau}_{FRET}$ and x $e^{-t/\tau}_{0}$ are the interacting and non-interacting donor molecules decay and a and b are the intensity factors of the interacting and non-interacting components. A more accurate calculation of the interacting and non-interacting protein populations can be obtained by assuming the interaction distances to be constant throughout the cell (Peter et al., 2005). Figure 3.8(A) shows the variation in the interacting donor population throughout the cell (a1%).

It was important to demonstrate that the abundance of splicing factors in the speckles did not lead to false positives. Previously there has been no biochemical evidence to suggest a direct interaction between U170K and U2AF35, instead it is proposed that SR proteins bridge these factors (Wu and Maniatis, 1993). This provided a negative control that could be analysed by FLIM. As expected the cotransfection of EGFP-U170K and mCherry-U2AF35 did not result in significant FRET being observed (Figure 3.8(B)). These data demonstrate that U170K shows specificity for the factors that it interacts with in the speckles and that the abundance of fluorescently tagged proteins in the speckles is not sufficient to induce FRET.





Images in panel B were taken by David Lleres (Dundee)

Figure 3.10: (A) FRET between EGFP-U170K and mCherry-SF2/ASF measured by FLIM. HeLa cells were transfected with EGFP-U170K and cotransfected with either mCherry-C1 or mCherry-SF2/ASF. Confocal image of transfected cells and FLIM image of same cells, in which mean fluorescent lifetime is shown in false colour. The colour scale with the respective lifetimes (in picoseconds) is indicated. The FRET Efficiencies were calculated by fixing the t2 lifetime to the average lifetime value for EGFP-U170K cotransfected with mCherry-C1. The FRET efficiencies are shown in either continuous or discrete false colour. The colour scale with the respective efficiencies (%) is indicated. The % of interacting population (a1) was calculated by fixing both the t1 and t2 populations. **(B) Non-significant FRET is observed between EGFP-U170K and mCherry-U2AF35.** HeLa cells were transfected with EGFP-U170K and cotransfected with mCherry-U2AF35. Cells were analysed exactly as described for panel (A)

3.8 Localisation of the U170K-SF2/ASF interaction in transcriptionally repressed cells

Several lines of evidence point to speckles functioning as storage/assembly/modification compartments that can supply splicing factors to active transcription sites (Misteli, 2000). Therefore, I determined whether treatment with DRB affected the localisation of the interaction between EGFP-U170K and mCherry-SF2/ASF.

Figure 3.10(A) shows the images captured by FLIM microscopy following treatment with DRB. This treatment was shown not to affect the fluorescent lifetime of donor fluorophore. The false colour images show that FRET can be observed upon cotransfection of EGFP-U170K and mCherry-SF2/ASF. The strongest FRET efficiencies were observed in the enlarged rounded upon speckles and certain regions of the nucleoplasm showed non-significant FRET. These variations between the speckles and nucleoplasm were analysed for a population of cells and compared to untreated cells. Figure 3.10(B) shows that the FRET efficiency between EGFP-U170K and mCherry-SF2/ASF is not altered by DRB when every pixel in the cell is analysed. However, while there is increased FRET in the speckles compared to the nucleoplasm in untreated cells this distribution is more pronounced upon treatment with DRB. A t-test has been applied to demonstrate that a significant decrease is observed in the nucleoplasm, but not the speckles, upon treatment with DRB.

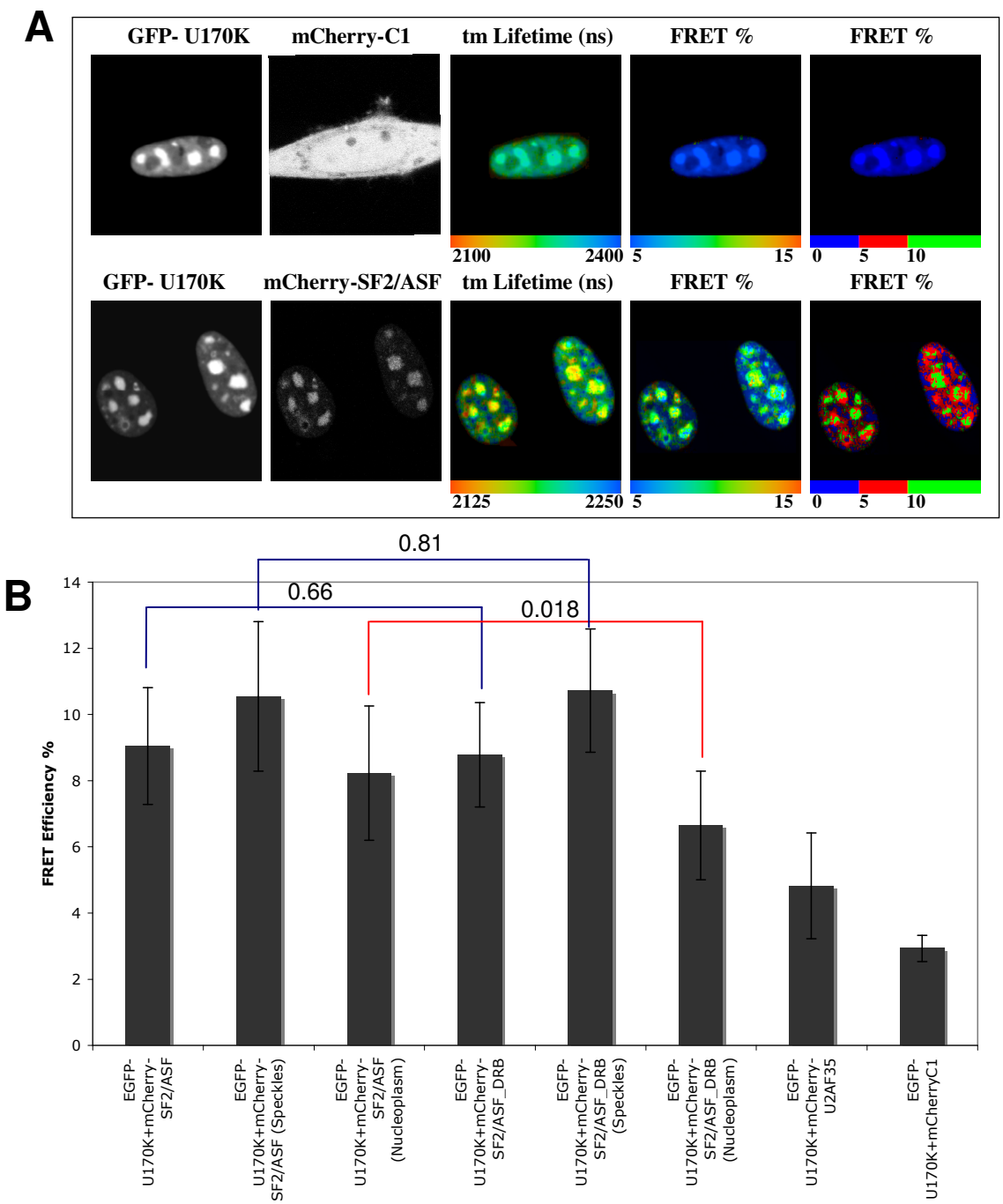


Figure 3.11: FRET between EGFP-U170K and mCherry-SF2/ASF, in the presence of DRB, measured by FLIM. . Experiments were performed exactly as described for figure 3.8 except cells were treated with 25 µg/ml of DRB for 2 hours before images were taken. **(B) FRET efficiencies determined by FLIM for interaction of SF2/ASF with U170K in the presence and absence of DRB.** FRET efficiencies between the EGFP and mCherry tagged pairs were determined by fixing the t2 to the mean lifetime for the donor cotransfected with mCherry-C1. Plot of mean FRET efficiencies +/- SD for 9 to 20 cells. To measure the FRET efficiency in the speckles and nucleoplasm a region characteristic of each was selected for each cell.

3.9 U170K and SF2/ASF interact in immunoprecipitation assays

It has previously been shown that recombinant U170K and SF2/ASF expressed in baculovirus can interact in GST-pull-down assays (Wu and Maniatis, 1993). To confirm that the endogenous proteins can interact I carried out co-immunoprecipitation (Co-IP) experiments using human 293T cells extracts that were immunoprecipitated with a monoclonal antibody against U170K. The immunoprecipitated proteins were separated on SDS-PAGE, blotted and detected with an anti-SF2/ASF antibody. Figure 3.10(A) shows that U170K was able to pull down SF2/ASF and that this interaction is not mediated by RNA.

To add further evidence that the interaction between U170K and SF2/ASF is not abolished by DRB treatment the Co-IP experiments were repeated but the cells were treated with DRB prior to making the lysates. Figure 3.10(B) shows that DRB has no effect on the ability of T7-U170K to pull down SF2/ASF. This Co-IP experiment was performed using an epitope tagged version of U170K to increase the efficiency of the immunoprecipitation. This adds *in vitro* data to confirm the interactions observed by FRET microscopy.

In conclusion I have shown that U170K and SF2/ASF interact in the speckles and the nucleoplasm even in transcriptionally repressed cells. The fact that the interaction in speckles becomes more pronounced upon treatment with DRB supports the view that speckles act as storage or assembly sites for splicing factors. The data also suggest that U170K and SF2/ASF form a complex in the speckles before being recruited to the spliceosome.

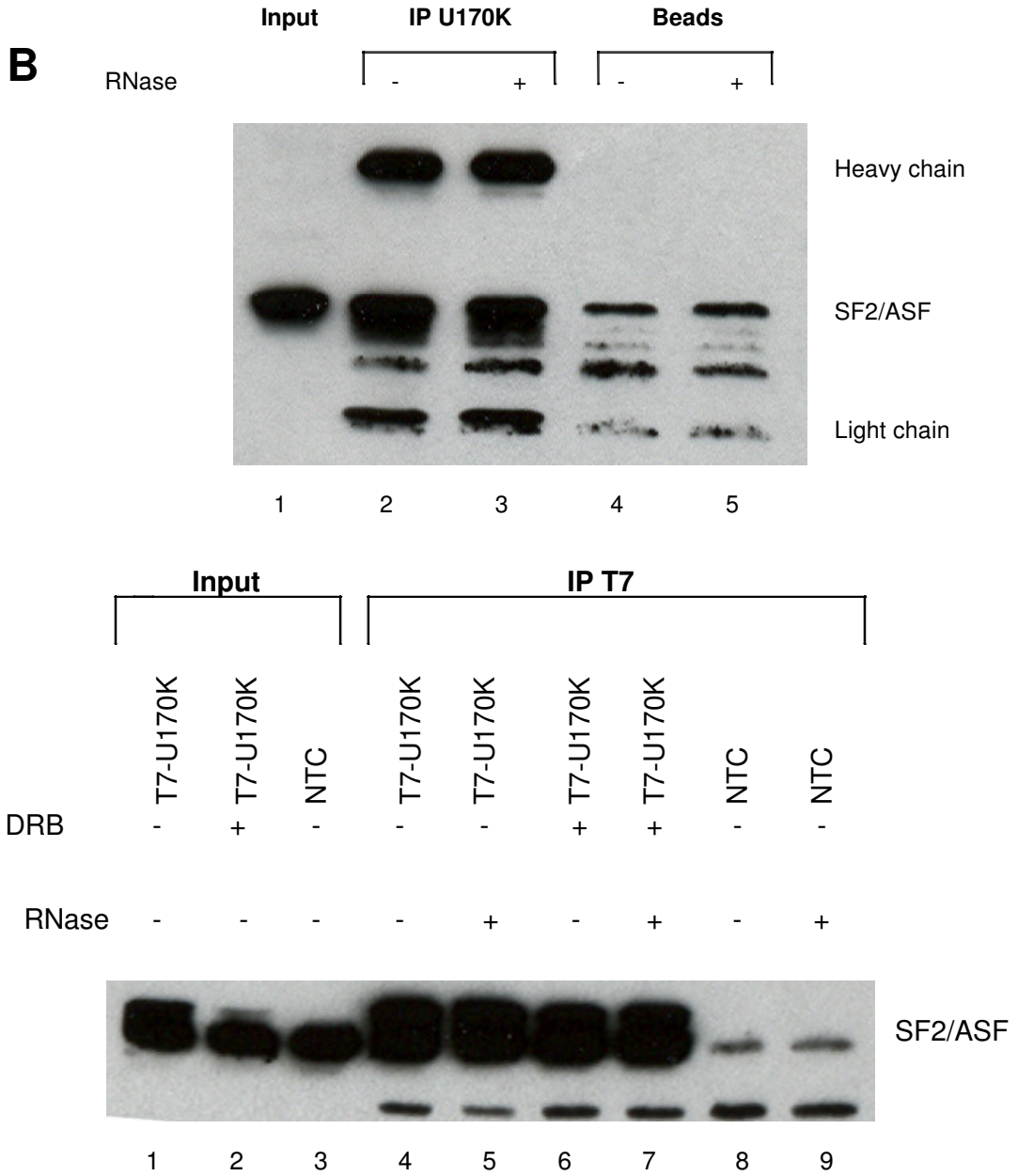


Figure 3.12: (A) U170K interacts with SF2/ASF in cultured mammalian cells. Extracts prepared from 293T were incubated with either anti-U170K antibody bound to sepharose beads (lanes 2 and 3) or sepharose beads alone (lanes 4 and 5). The bound proteins were analysed by Western blotting with mAb96 for detection of SF2/ASF. Alternatively the immunoprecipitate was treated with RNase (lanes 3 and 5). Lane 1 was loaded with 2% of the amount of extract used for each IP. **(B)The interaction between T7-U170K and SF2/ASF is not perturbed by DRB in cultured mammalian cells.** Extracts prepared from 293T cells either transiently transfected with pCG-T7-U170K (lanes 4 to 7) or mock transfected (lanes 8 and 9) were incubated with anti-T7 antibody bound to sepharose beads. 24 hours post transfection cells were treated for 2 hours with DRB (lanes 6 and 7). The bound proteins were analysed by Western blotting with anti-SF2/ASF antibody. Alternatively immunoprecipitates were treated with RNase (Lanes 5, 7 and 9). Lanes 1 to 3 were loaded with 2% of the amount of extract used for each IP.

3.10 FRET acceptor photobleaching demonstrates the interaction of SF2/ASF with U2AF35 in live cells

U2AF35 binds to the AG dinucleotide at the 3' splice site (Zhang et al., 1992; Merendino et al., 1999a; Wu et al., 1999; Zorio and Blumenthal, 1999) and plays a role in stabilising the binding of U2AF65 to the polypyrimidine tract (Guth et al., 1999b). In addition it has been demonstrated that U2AF35 is capable of interacting with SR proteins by the same *in vitro* approaches used to demonstrate the interaction between U170K and SR proteins (Wu and Maniatis, 1993). The interaction between U2AF35 and SR proteins is proposed to play two important roles in the cell. Firstly, SR proteins bound to exonic splicing enhancers (ESEs) interact with U2AF35 to promote complex assembly at the 3' splice site (Wang et al., 1995; Zuo and Maniatis, 1996; Graveley et al., 2001). Secondly, it is proposed that SR proteins can interact simultaneously with U2AF35 and U170K thereby bridging factors assembled on both 5' and 3' splice sites (Wu and Maniatis, 1993). This bridging can occur either across the exon or the intron and is therefore proposed to play a role in exon and intron definition.

I have already confirmed that U170K and SF2/ASF can interact together in live HeLa cells. To determine whether the interactions involved in exon and intron definition occur in living HeLa cells, I analysed the interaction between U2AF35 and SF2/ASF by FRET acceptor photobleaching. HeLa cells were cotransfected with ECFP-U2AF35 and EYFP-SF2/ASF and the donor intensities were monitored before and after photobleaching of the acceptor to calculate a FRET efficiency for this interaction. Figure 3.11 shows that there is significant FRET between ECFP-U2AF35 and EYFP-SF2/ASF and this FRET efficiency is very similar to that observed

between ECFP-U170K and EYFP-SF2/ASF. Figure 3.11 shows that the FRET efficiency between ECFP-U2AF35 and EYFP-SF2/ASF is not significantly affected by DRB treatment, as confirmed by a t-test.

FLIM microscopy demonstrated that significant FRET is not observed between EGFP-U170K and mCherry-U2AF35 (Figure 3.8(B)). This can be confirmed by acceptor photobleaching analysis as non-significant FRET is observed between ECFP-U170K and EYFP-U2AF35 (Figure 3.11). In addition, neither ECFP-U2AF35 nor ECFP-SF2/ASF showed significant FRET upon cotransfection with EYFP_NLS (Figure 3.11).

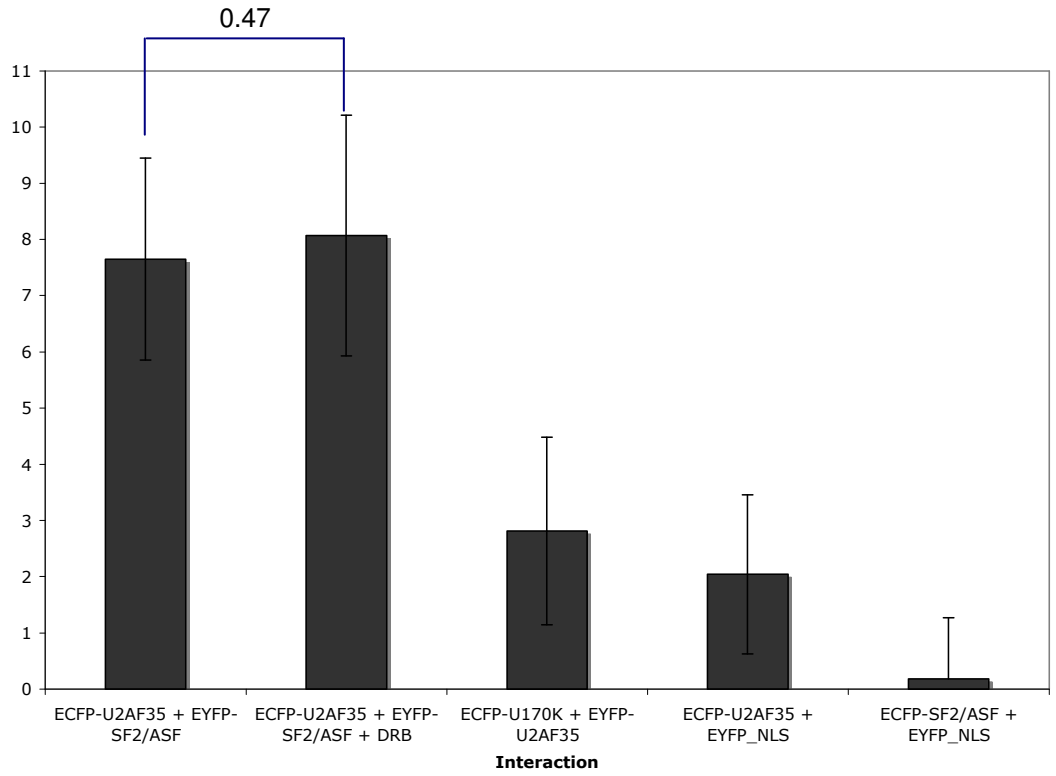


Figure 3.13: Effect of DRB on interaction between ECFP-U2AF35 and EYFP-SF2/ASF. Plot of FRET efficiencies (average for 7 to 27 cells) between ECFP + EYFP pairs measured by FRET acceptor photobleaching. Mean +/- SD.

3.11 FLIM demonstrates the interaction between U2AF35 and SF2/ASF occurs predominantly in speckles

The precise distribution of the interactions between EGFP-U2AF35 and mCherry-SF2/ASF were mapped using FLIM microscopy. Cotransfection of EGFP-U2AF35 and mCherry-SF2/ASF results in a shortening of the donor fluorescent lifetime. The false colour images shown in figure 3.12 depicting the FRET efficiencies in either continuous or discrete colour show mCherry-SF2/ASF interacts with EGFP-U2AF35 in a similar pattern to that observed for its interaction with EGFP-U170K. Significant FRET is observed in the nucleoplasm but the strongest regions of FRET are observed in the speckles. This is true of both DRB treated and untreated cells (Figure 3.12 middle and lower panels).

These differences in FRET efficiency were quantified for a population of cells. As was previously described for the interaction of EGFP-U170K and mCherry-SF2/ASF, a t-test has been applied to demonstrate that a significant decrease in FRET efficiency is observed in the nucleoplasm but not the speckles upon treatment with DRB (Figure 3.13).

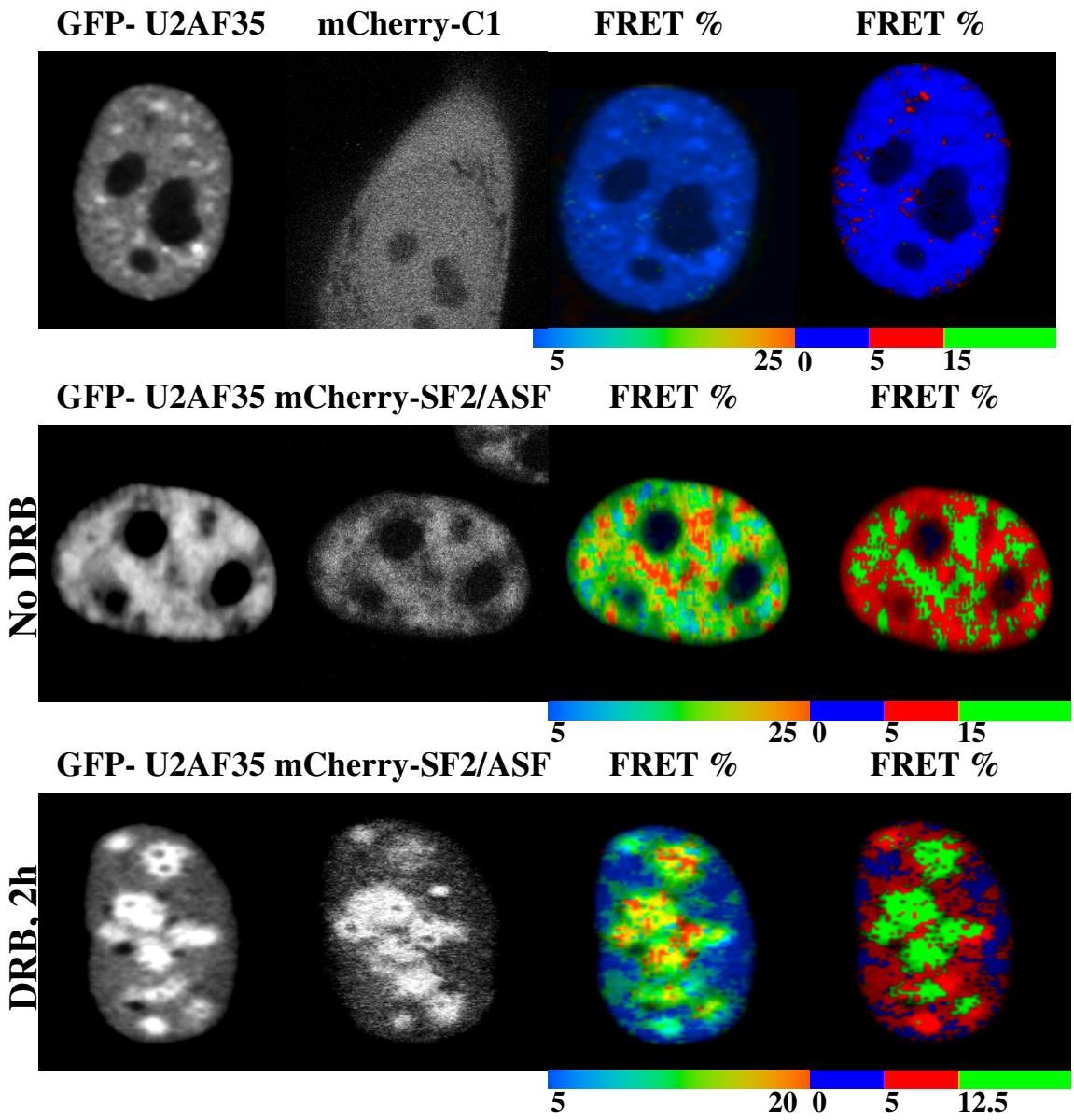


Figure 3.14: FRET between U2AF35 and SF2/ASF measured by FLIM. HeLa cells were transfected with EGFP-U2AF35 and cotransfected with either mCherry-C1 or mCherry-SF2/ASF. Confocal images of transfected cells and FLIM images of the same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies were calculated by fixing the t2 lifetime to the average lifetime value for EGFP-U2AF35 cotransfected with mCherry-C1. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP-U2AF35 + mCherry-C1 (Middle) EGFP-U2AF35 + mCherry-SF2/ASF (Bottom) EGFP-U2AF35 + mCherry-SF2/ASF, cells treated with 25 µg/ml DRB for two hours before imaging.

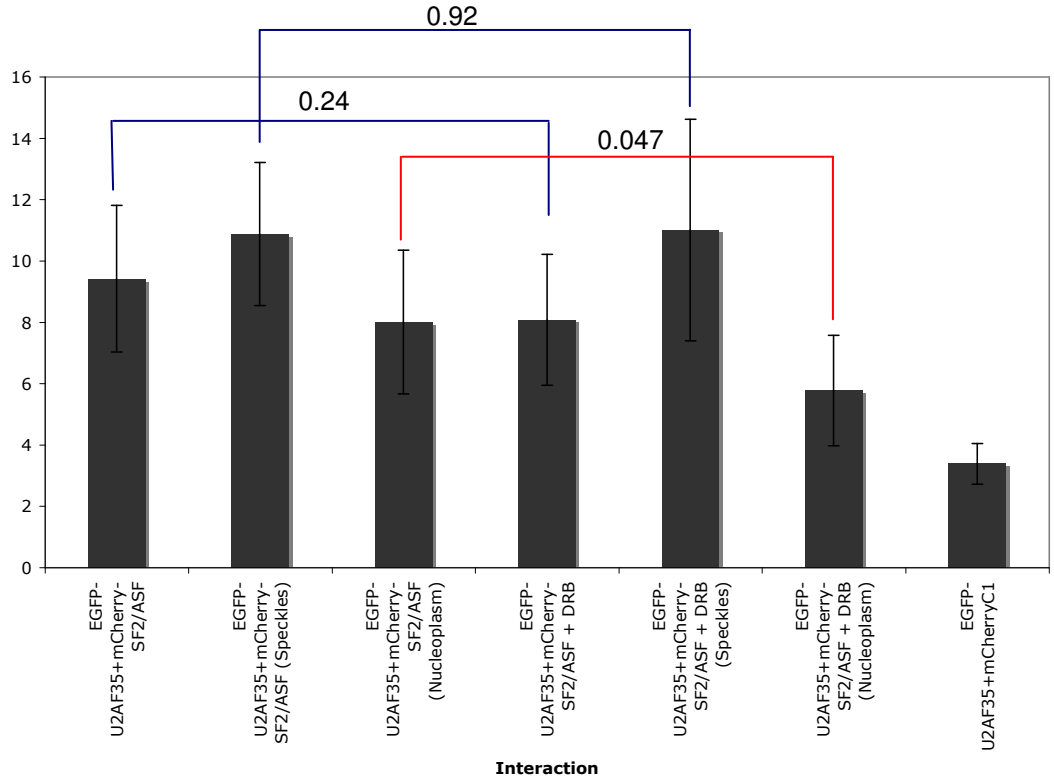


Figure 3.15: FRET efficiencies determined by FLIM for interaction of SF2/ASF with U2AF35 in the presence and absence of DRB. FRET efficiencies between the EGFP and mCherry tagged pairs were determined by fixing the t2 to the mean lifetime for the donor cotransfected with mCherry-C1. Plot of mean FRET efficiencies +/- SD for 7 to 12 cells. To measure the FRET efficiency in the speckles and nucleoplasm a region characteristic of each was selected for each cell.

3.12 U2AF35 and SF2/ASF interact in immunoprecipitation assays

The interaction between U2AF35 and SF2/ASF was confirmed by Co-IP assays. 293T cells were either transfected with EGFP-U2AF35 or mock transfected. Cell lysates were immunoprecipitated with an anti-GFP antibody. The immunoprecipitated proteins were then separated on SDS-PAGE, blotted and detected with an anti-SF2/ASF antibody. Figure 3.14 shows that endogenous SF2/ASF interacts with EGFP-U2AF35. Treatment with RNaseA demonstrates that RNA does not mediate this interaction.

In conclusion I have used FRET acceptor photobleaching and FLIM microscopy to demonstrate the interactions involved in exon and intron definition occur in living HeLa cells. The inhibition of transcription and therefore splicing did not affect the observed interactions demonstrating they are not exclusively cotranscriptional.

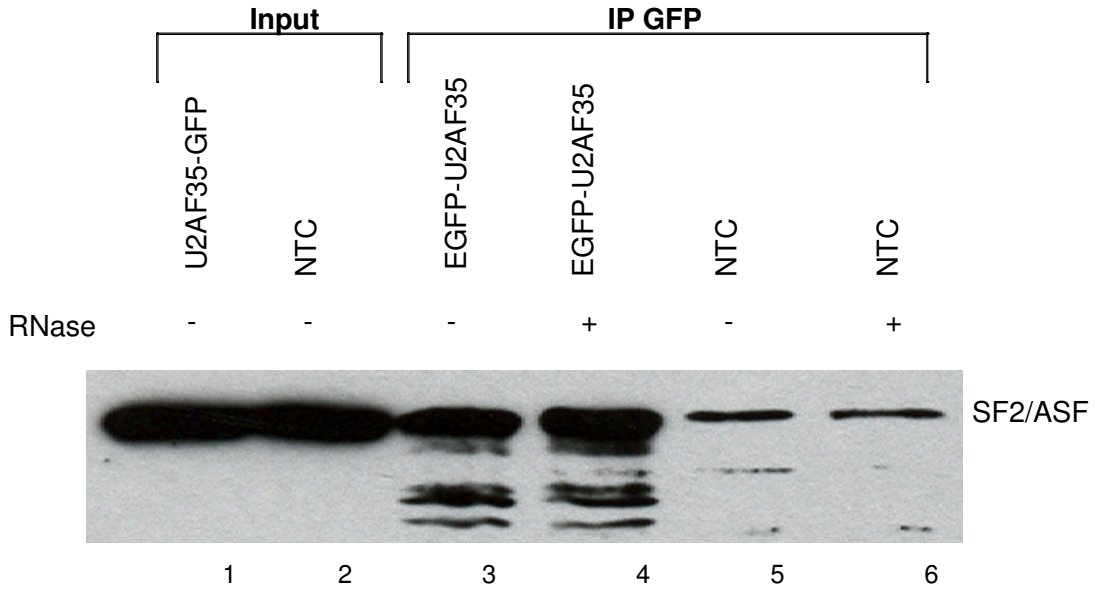


Figure 3.16: EGFP-U2AF35 interacts with SF2/ASF in cultured mammalian cells. IP assays with EGFP-U2AF35. Extracts prepared from 293T cells either transiently transfected with EGFP-U2AF35 (lanes 3 and 4) or mock-transfected (lanes 5 and 6) were incubated with anti-GFP antibody bound to sepharose beads. The bound proteins were separated on an SDS 12% polyacrylamide gel and analysed by Western blotting with mAb96. Lanes 1 and 2 were loaded with 2% of the amount of extract used for each IP. Alternatively the immunoprecipitate was treated with RNAase (lanes 4 and 6). NTC = Non-transfected cells.

3.13 Several SR proteins interact with U170K and U2AF35

SR proteins are functionally redundant in the splicing of some introns. However, several differences in the ability of these proteins to regulate alternative splicing, as well as the ability of individual SR proteins to commit different pre-mRNAs to the splicing pathway suggested that individual SR proteins have unique functions in splicing regulation (Caceres et al., 1994; Chandler et al., 1997; Fu, 1993; Wang and Manley, 1995).

In vitro experiments have previously shown both SF2/ASF and SC35 are capable of interacting with U170K and U2AF35 (Wu and Maniatis, 1993). Here, I have extended this study to show that both SC35 and SRp20 also interact with U170K and U2AF35 in live HeLa cells. The FRET efficiencies obtained from the acceptor photobleaching analysis of these interactions are shown in figure 3.15(A). The FRET efficiencies for the interaction of individual SR proteins with components of the 5' (U170K) or 3' (U2AF35) splice site were shown to be similar. It was also demonstrated that DRB does not affect the FRET efficiencies for the interaction of SC35 with U2AF35. Interestingly, a significant increase in FRET efficiency is observed for the interaction of U170K with SC35 upon treatment with DRB. The FRET efficiencies in FRET acceptor photobleaching experiments were measured in the speckles therefore this may reflect the fact that when transcription is blocked splicing factors are not recruited to active sites of splicing in the nucleoplasm, resulting in a higher FRET efficiency in the speckles. The increased FRET efficiency upon treatment with DRB may not have been observed in FLIM experiments due the fact that the FRET efficiencies are measured for the whole cell and not just the

speckles. The interaction of SRp20 with U170K and U2AF35 has not been tested in the presence of DRB as I have demonstrated that both shuttling (SF2/ASF) and non-shuttling SR-proteins (SC35) form complexes with U170K and U2AF35 in the absence of ongoing transcription.

FLIM microscopy was employed to map the protein-protein interaction sites of mCherry-SC35 with EGFP-U170K and EGFP-U2AF35. Figures 3.16 and 3.17 show that at least in certain cells the highest FRET efficiencies do not correspond with regions of speckles, as was the case with SF2/ASF, but are instead observed in areas of the nucleoplasm. A similar pattern was observed upon treatment with DRB making it unlikely that these regions of high FRET correspond to regions of active transcription and splicing. By measuring the average FRET efficiencies for a number of cells it was shown that the FRET efficiencies measured by acceptor photobleaching or FLIM are very similar (Figure 3.15). The interaction of SC35 with U170K and U2AF35 has also been confirmed by Co-IP experiments (Figure 3.18).

In conclusion I have shown that the ability of SR proteins to interact with components at the 5' and 3' splice site in live HeLa cells is not confined to SF2/ASF. The interactions patterns of a shuttling, SF2/ASF and nonshuttling SR protein, SC35 have been compared. While neither are sensitive to DRB treatment there appears to be differences in the localisation of their protein interactions within the nucleus.

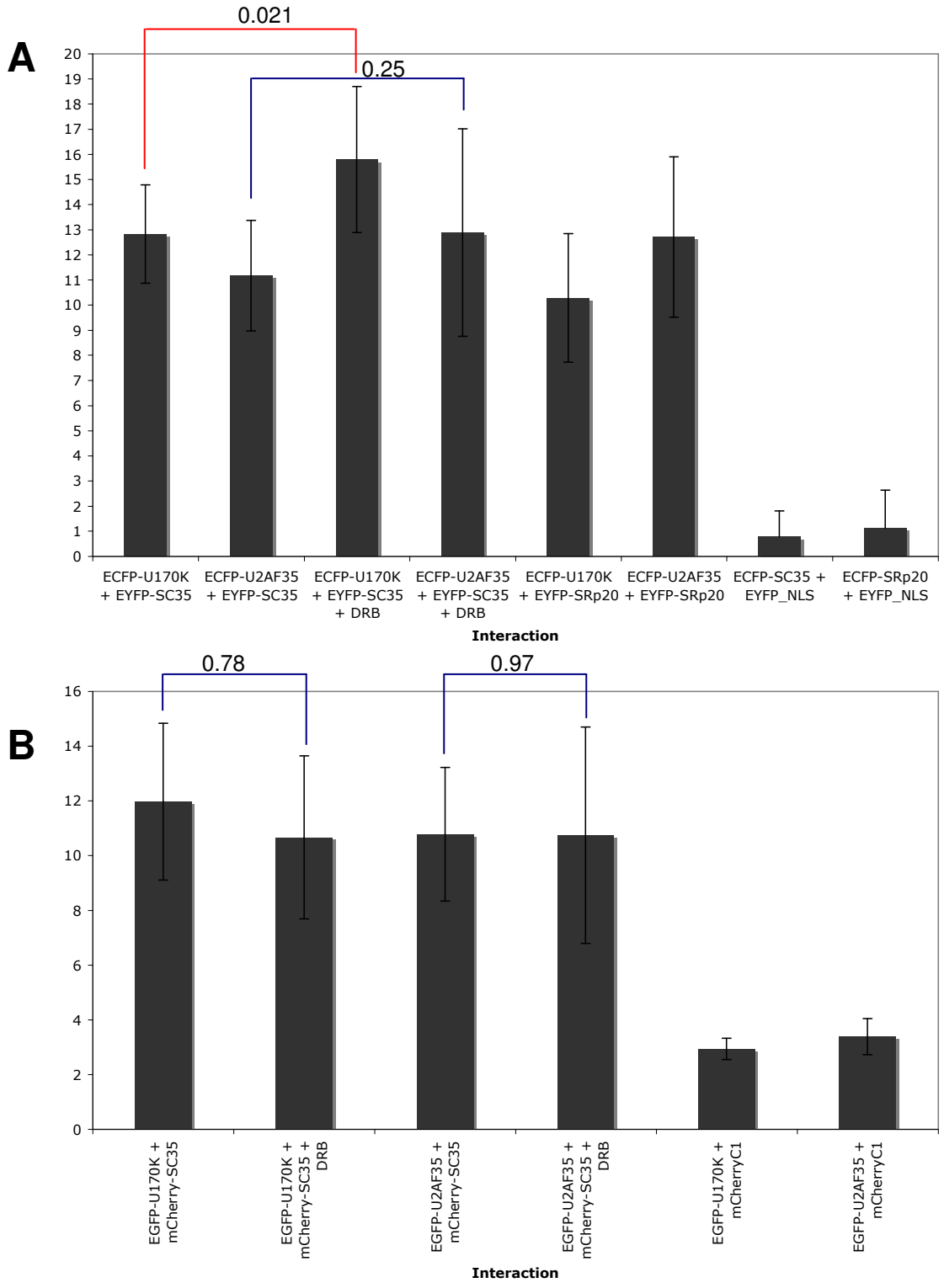


Figure 3.17: (A) SRp20 and SC35 interact with U170K and U2AF35. Plot of FRET efficiencies (average for 7 to 14 cells) between CFP + YFP pairs measured by FRET acceptor photobleaching. Mean +/- SD. **(B) FRET efficiencies determined by FLIM for interaction of SC35 with U170K and U2AF35 in the presence and absence of DRB.** FRET efficiencies between the EGFP and mCherry tagged pairs were determined by fixing the t2 to the mean lifetime for the donor cotransfected with mCherry C1. Plot of mean FRET efficiencies +/- SD for 8 to 18 cells.

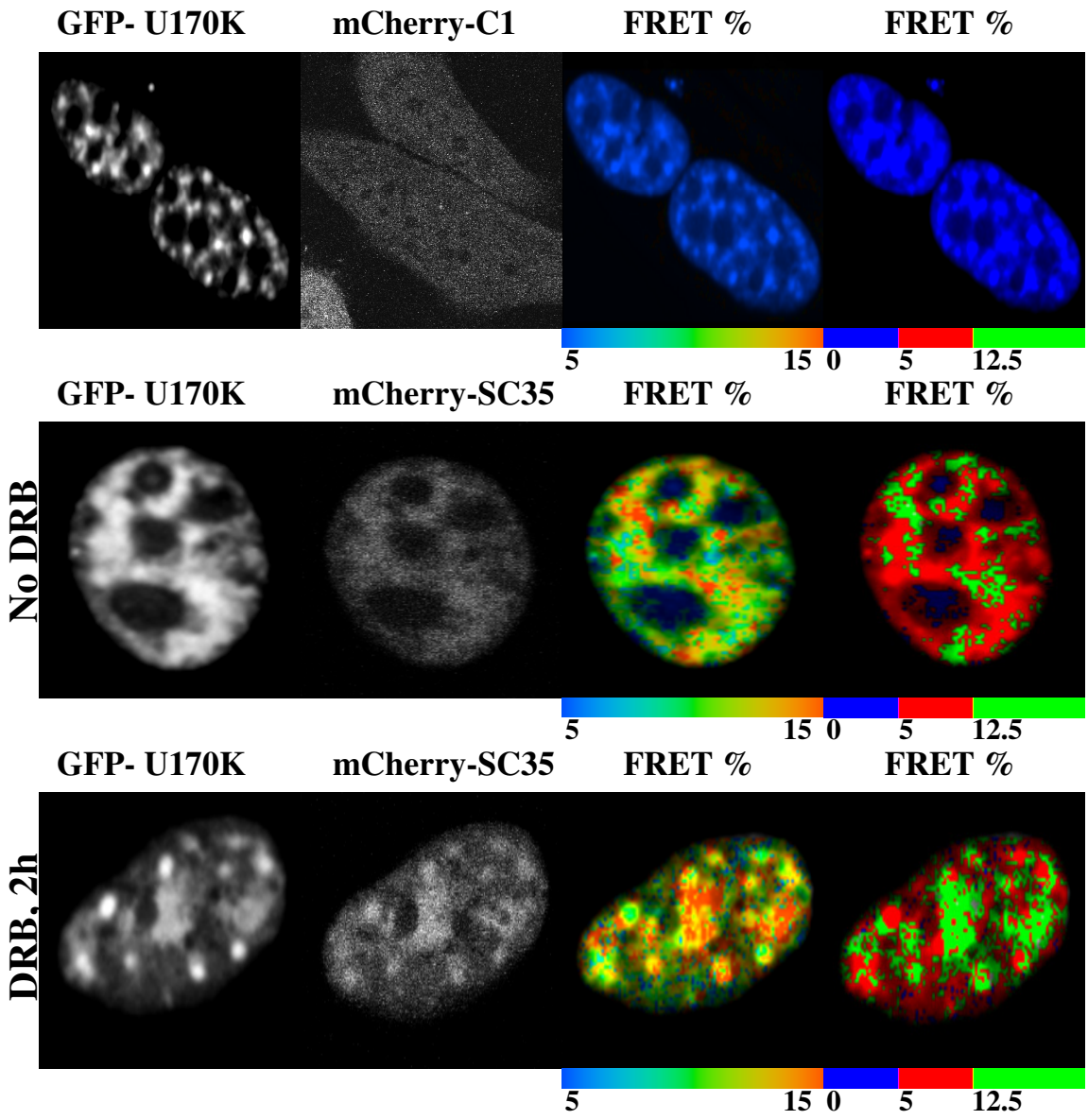


Figure 3.18: FRET between U170K and SC35 measured by FLIM. HeLa cells were transfected with EGFP-U170K and cotransfected with either mCherry-C1 or mCherry-SC35. Confocal image of transfected cells and FLIM image of same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies were calculated by fixing the t2 lifetime to the average lifetime value for EGFP-U170K cotransfected with mCherry-C1. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP-U170K + mCherry-C1 (Middle) EGFP-U170K + mCherry-SC35 (Bottom) EGFP-U170K + mCherry-SC35, cells treated with 25 µg/ml DRB for two hours before imaging

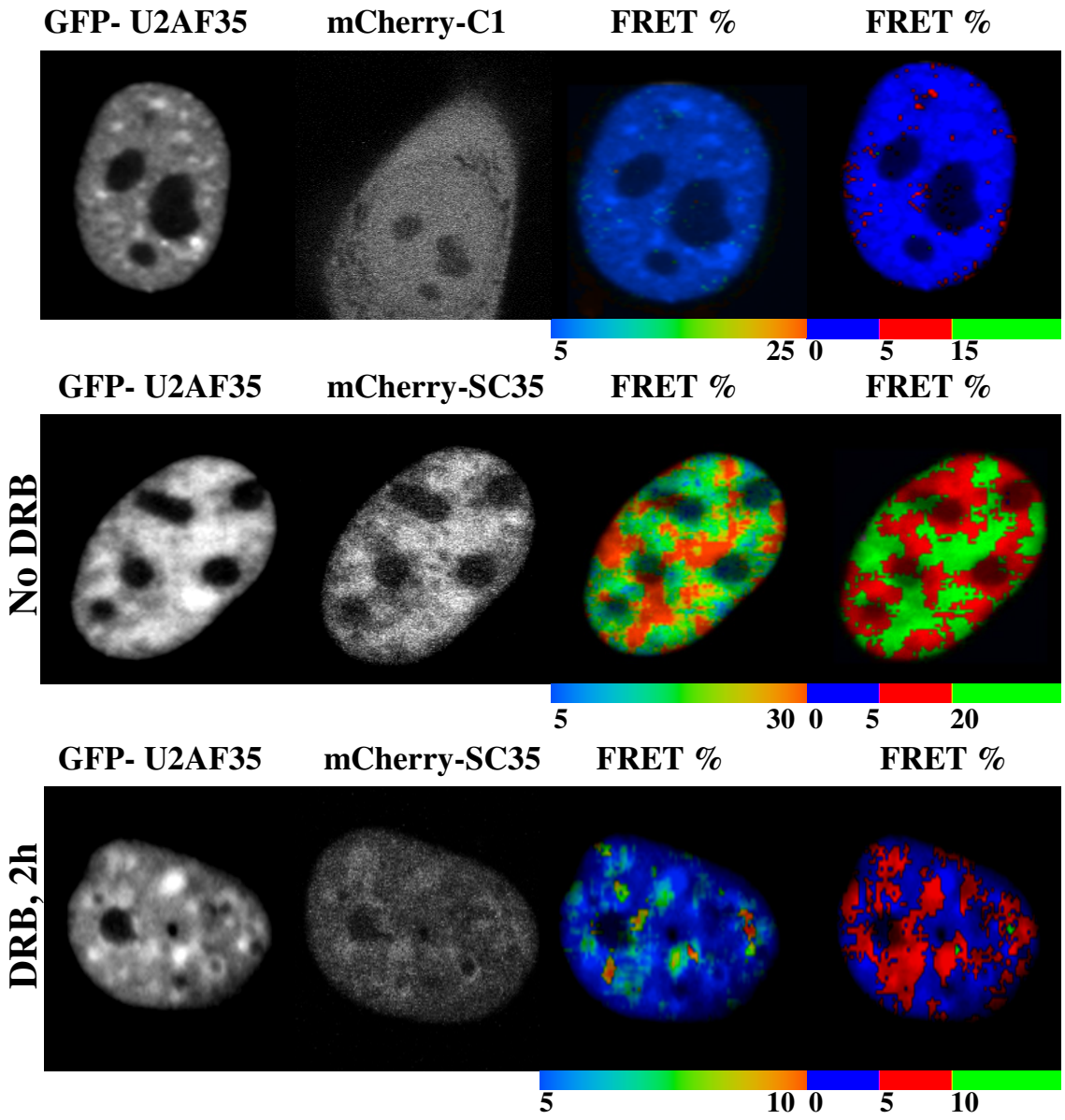


Figure 3.19: FRET between U2AF35 and SC35 measured by FLIM. HeLa cells were transfected with EGFP-U2AF35 and cotransfected with either mCherry-C1 or mCherry-SC35. Confocal image of transfected cells and FLIM image of same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies were calculated by fixing the t2 lifetime to the average lifetime value for EGFP-U2AF35 cotransfected with mCherry-C1. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP-U2AF35 + mCherry-C1 (Middle) EGFP-U2AF35 + mCherry-SC35 (Bottom) EGFP-U2AF35 + mCherry-SC35, cells treated with 25 µg/ml DRB for two hours before imaging

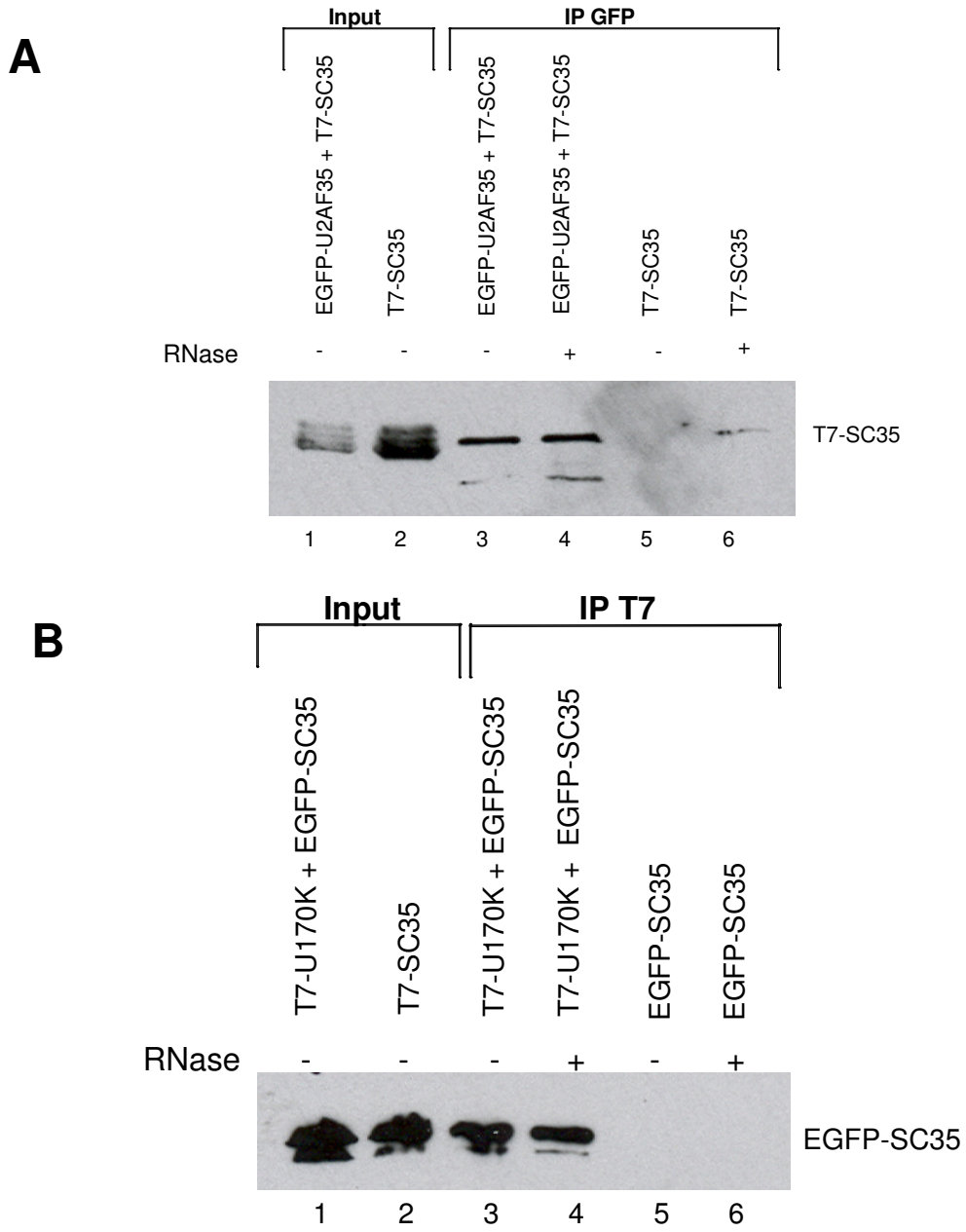


Figure 3.20: (A) EGFP-U2AF35 interacts with T7-SC35 in cultured mammalian cells. IP assays with EGFP-U2AF35. Extracts prepared from 293T cells either transiently transfected with EGFP-U2AF35 and pCG-T7-SC35 (lanes 3 and 4) or pCG-T7-SC35 (lanes 5 and 6) were incubated with anti-GFP antibody bound to sepharose beads. The bound proteins were analysed by Western blotting with T7 antibody. Alternatively the immunoprecipitate was treated with RNAase (lanes 4 and 6). Lanes 1 and 2 were loaded with 2% of the amount of extract used for each IP. **(B) EGFP-SC35 interacts with T7-U170K in cultured mammalian cells.** Extracts prepared from 293T cells either transiently transfected with EGFP-SC35 and pCG-T7-U170K (lanes 3 and 4) or EGFP-SC35 (lanes 5 and 6) were incubated with anti-T7 antibody bound to sepharose beads. The bound proteins were analysed by Western blotting with anti-GFP antibody. Alternatively the immunoprecipitate was treated with RNAase (lanes 4 and 6). Lanes 1 and 2 were loaded with 2% of the amount of extract used for each IP.

3.14 Self-interactions of SR proteins

Previously, *in vitro* data and yeast two-hybrid analysis have shown that SC35 is capable of interacting with itself and with SF2/ASF (Wu and Maniatis, 1993). In addition, the Drosophila SR-related proteins Transformer (Tra) and Transformer-2 (Tra-2) have been shown to interact with each other, with themselves, and with SC35 and SF2/ASF (Amrein et al., 1994; Wu and Maniatis, 1993). I have studied the interactions between SR proteins using acceptor photobleaching and FLIM microscopy to confirm SC35 interacts with SF2/ASF and identify a novel self-interaction of SF2/ASF.

Figure 3.19(A) shows cotransfection of ECFP-SF2/ASF with either EYFP-SF2/ASF or EYFP-SC35 resulted in significant FRET, with similar FRET efficiencies being measured by acceptor photobleaching for both interactions. FLIM microscopy revealed the distribution of these interactions within the nucleus are similar to those observed for the interaction of SF2/ASF with EGFP-U170K and EGFP-U2AF35 with the highest FRET efficiencies occurring in the speckles (Figure 3.20). The FRET efficiencies measured by FLIM were quantified for a number of cells to confirm significant FRET could be measured by both acceptor photobleaching and FLIM microscopy for the interaction of SF2/ASF with SC35 and with itself (Figure 3.19).

This novel self-interaction of SF2/ASF has been confirmed by Co-IP experiments in 293T cells. Figure 3.21 shows that immunoprecipitated EGFP-SF2/ASF is capable of pulling down endogenous SF2/ASF.

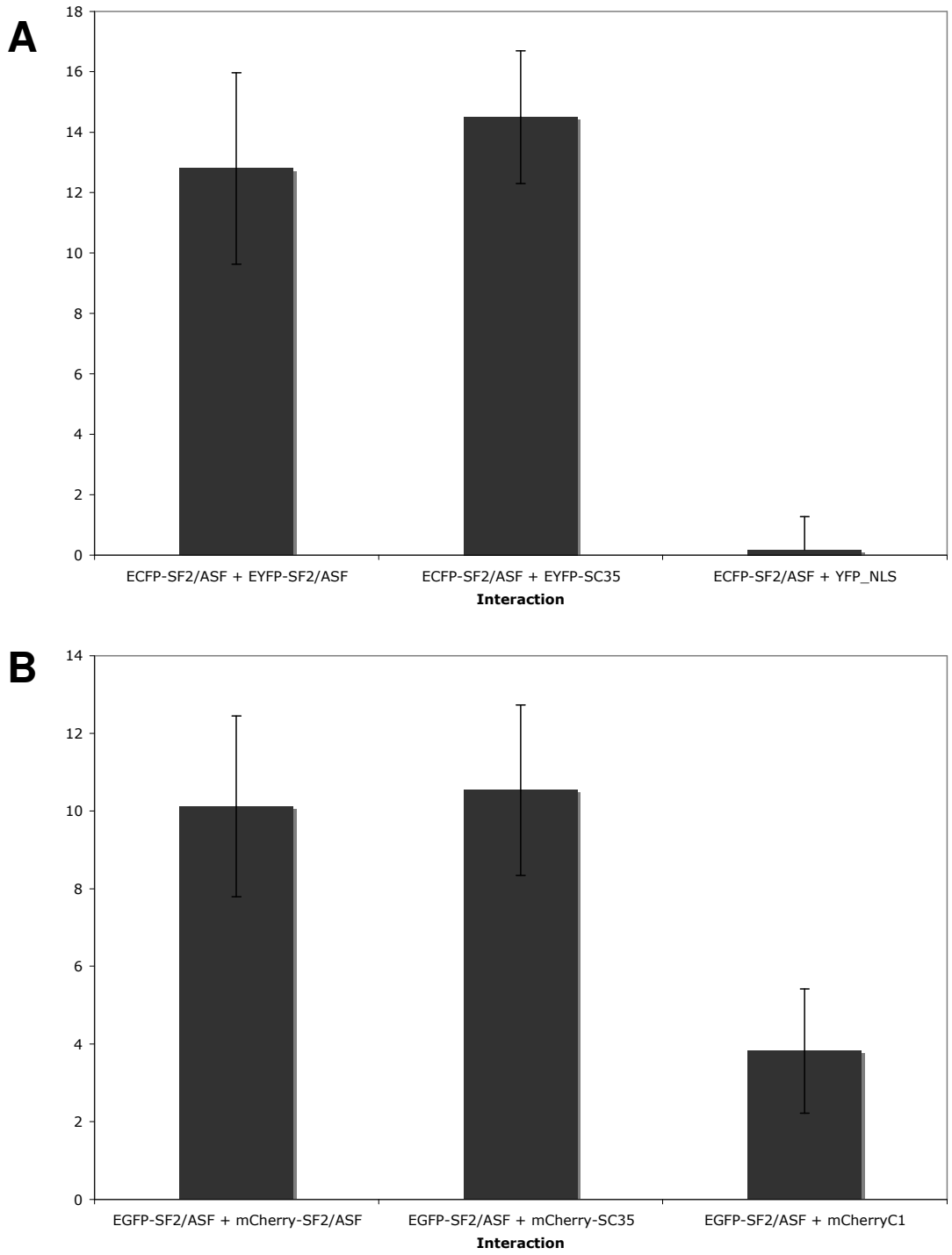


Figure 3.21: (A) SF2/ASF interacts with SC35 and is capable of interacting with itself. Plot of FRET efficiencies (average for 10-12 cells) between CFP + YFP pairs measured by FRET acceptor photobleaching. Mean +/- SD. **(B) FRET efficiencies determined by FLIM for interaction of SF2/ASF with SC35 itself.** FRET efficiencies between the EGFP and mCherry tagged pairs were determined by fixing the t2 to the mean lifetime for the donor cotransfected with mCherry-C1. Plot of mean FRET efficiencies +/- SD for 11-12 cells.

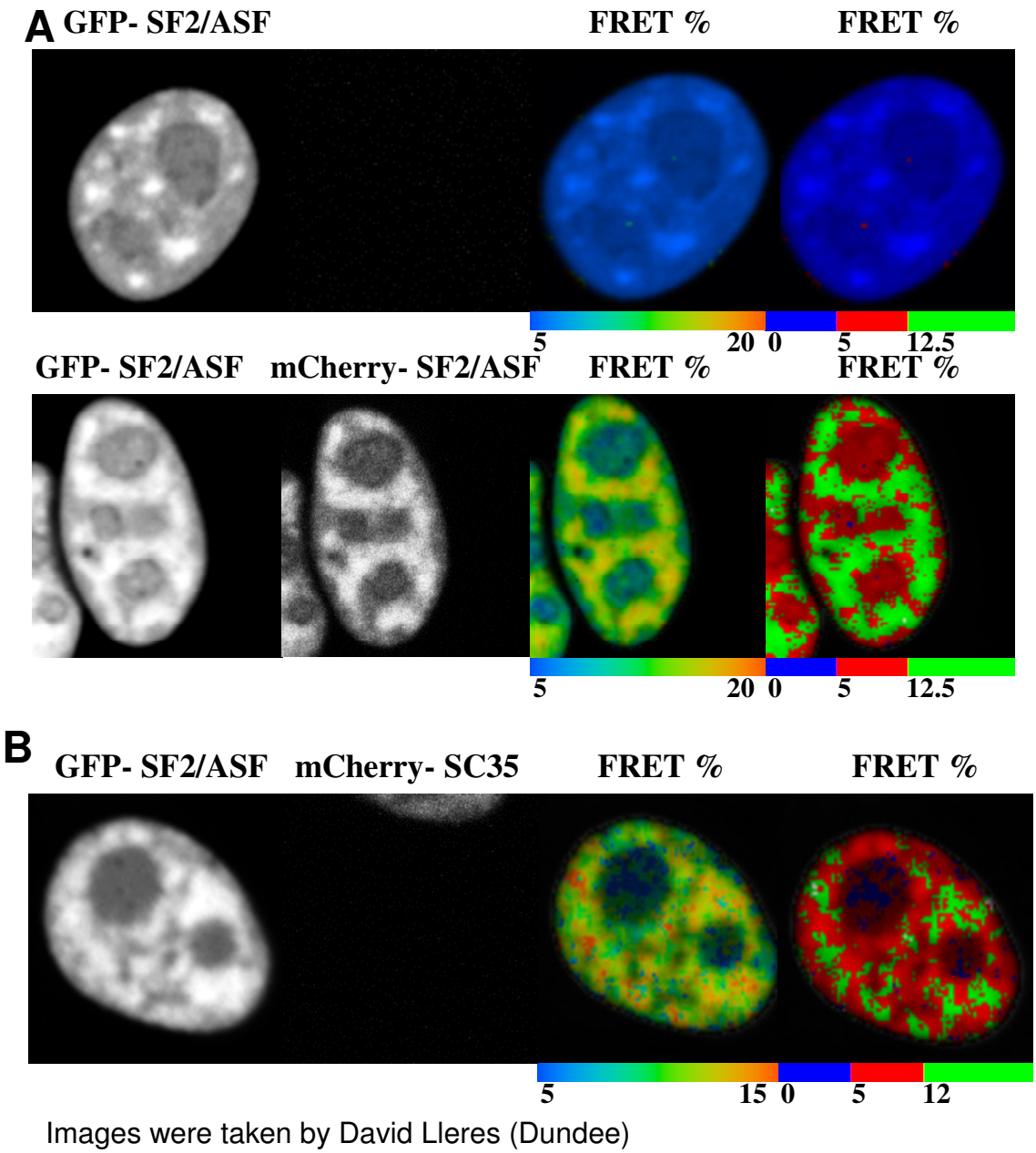


Figure 3.22: (A) FRET between SF2/ASF and SF2/ASF measured by FLIM. HeLa cells were transfected with EGFP-SF2/ASF or cotransfected mCherry-SF2/ASF. Confocal image of transfected cells and FLIM image of same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies were calculated by fixing the t2 lifetime to the average lifetime value for EGFP-SF2/ASF cotransfected with mCherry-C1. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP-SF2/ASF (Bottom) EGFP-SF2/ASF + mCherry-SF2/ASF **(B) FRET between SF2/ASF and SC35 measured by FLIM** Experiments were performed as described in panel (A) except cells were cotransfected with EGFP-SF2/ASF and mCherry-SC35.

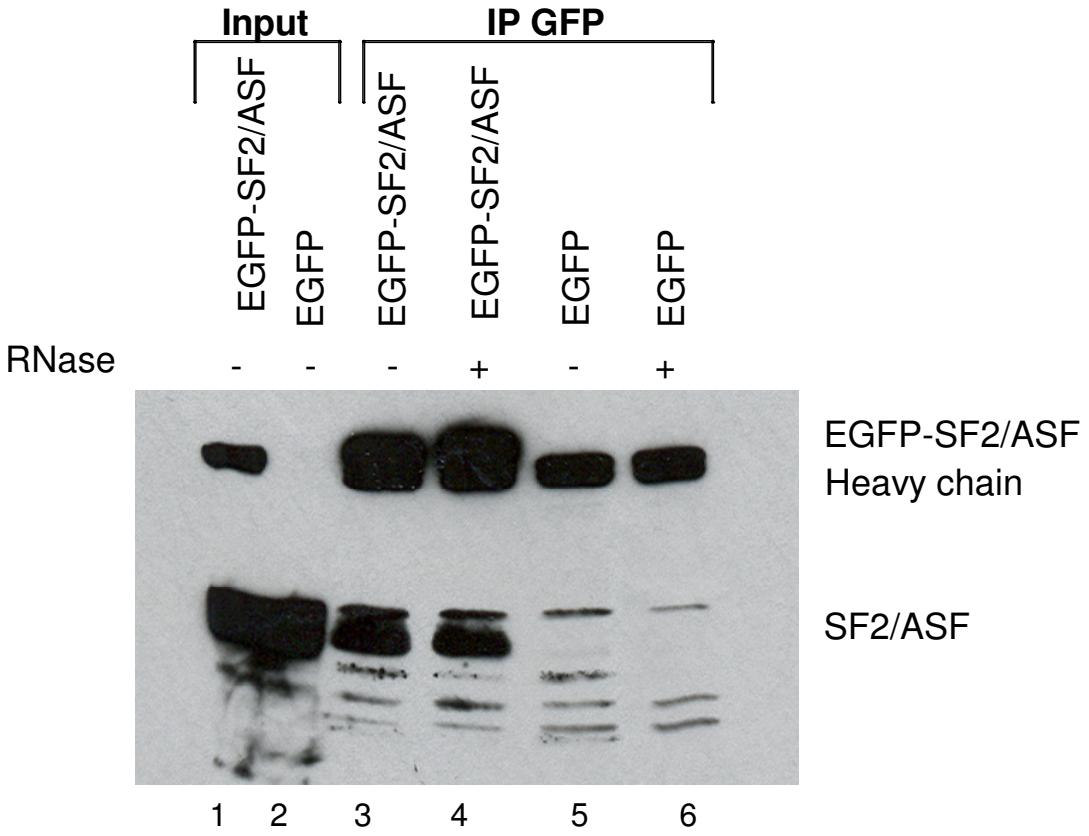


Figure 3.23: EGFP-SF2/ASF interacts with endogenous SF2/ASF in cultured mammalian cells. IP assays with EGFP-SF2/ASF. Extracts prepared from 293T cells either transiently transfected with EGFP-SF2/ASF (lanes 3 and 4) or EGFP-C1 (lanes 5 and 6) were incubated with EGFP antibody bound to sepharose beads. The bound proteins were separated on an SDS 12% polyacrylamide gel and analysed by Western blotting with mAb96. Alternatively the immunoprecipitate was treated with RNAase (lanes 4 and 6). Lanes 1 and 2 were loaded with 2% of the amount of extract used for each IP.

In conclusion I have employed FRET microscopy to confirm existing *in vitro* and yeast two-hybrid data on the interaction of SF2/ASF with SC35 and identified a novel ability of SF2/ASF to interact with itself. These interactions are important in determining how SR proteins can simultaneously interact with U170K and U2AF35 and contact the pre-mRNA through their RS domains at several stages during spliceosome assembly.

3.15 Mutational analysis of domains of SF2/ASF required for the interactions with U170K and SF2/ASF

Previous studies have suggested that RS domains participate in protein-protein interactions with other RS domain containing proteins. The RS domain of U2AF35 is required for interactions with Tra, Tra2 and SR proteins and U2AFΔRS is inactive in enhancer-dependent splicing (Zuo and Maniatis, 1996). Furthermore, a serine-arginine rich domain of U170K has been shown to be necessary and sufficient for SF2/ASF binding by yeast-two hybrid and far western analysis (Cao and Garcia-Blanco, 1998). The RS domain of SF2/ASF has been shown to be necessary but not sufficient for binding to U170K *in vitro* (Xiao and Manley, 1997; Jamison et al., 1995). I have used FRET microscopy to study the protein-protein interactions of a mutant of SF2/ASF lacking the C-terminal RS domain (SF2/ASFΔRS) in live HeLa cells. Significant FRET was observed by acceptor photobleaching and FLIM for the interaction of SF2/ASFΔRS with U2AF35 and U170K and the FRET efficiencies were similar to those measured for the wild type protein (Figure 3.22).

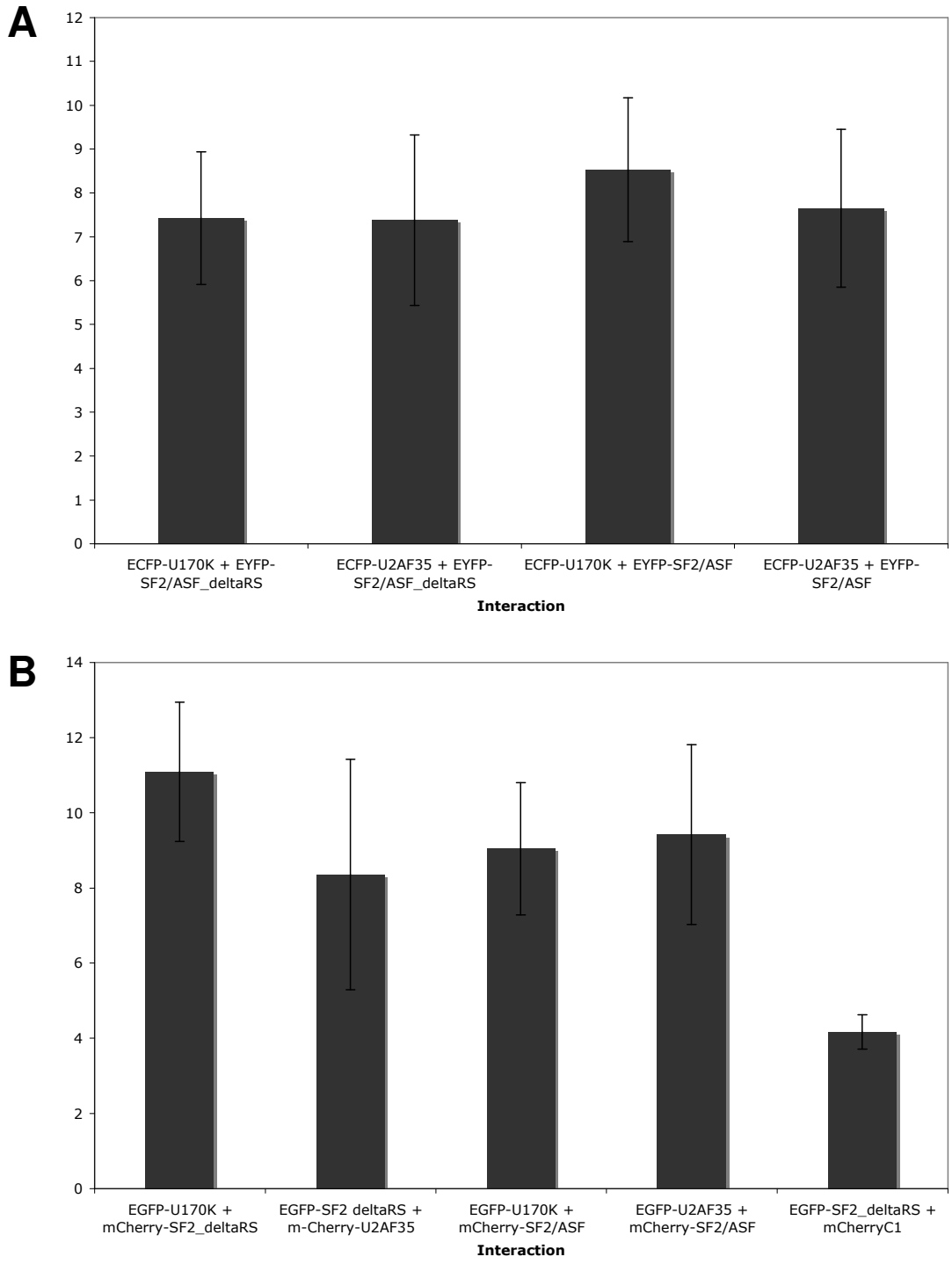


Figure 3.24: (A) FRET efficiencies for interaction of SF2/ASFΔRS with U170K and U2AF35. Plot of FRET efficiencies (average for 8 cells) between CFP + YFP pairs measured by FRET acceptor photobleaching. Mean +/- SD. **(B) FRET efficiencies determined by FLIM for interaction of SF2ΔRS with U170K and U2AF35.** FRET efficiencies between the EGFP and mCherry tagged pairs were determined by fixing the t2 to the mean lifetime for the donor cotransfected with mCherry C1. Plot of mean FRET efficiencies +/- SD for 7 cells.

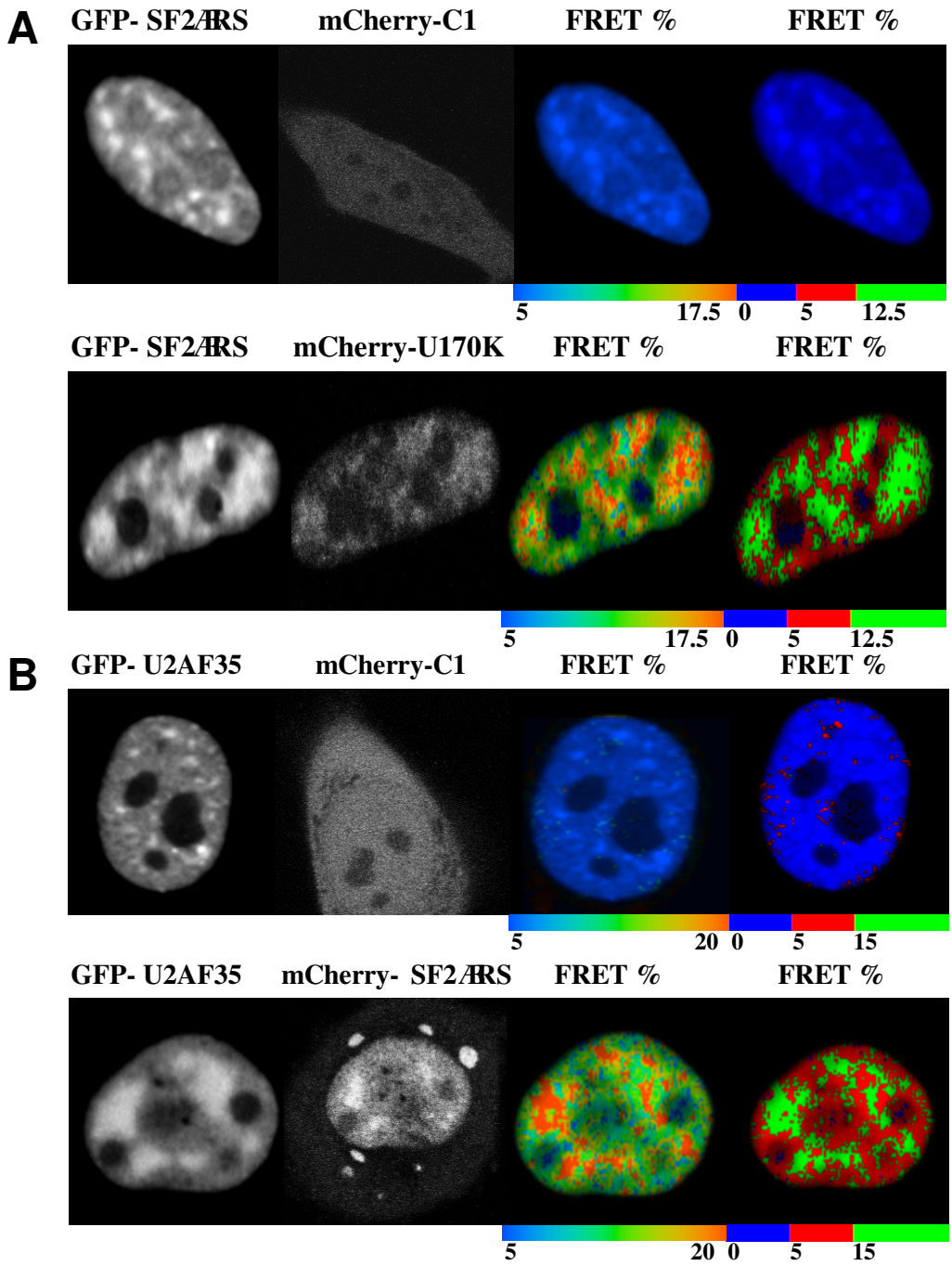


Figure 3.25: (A) FRET measured by FLIM for interaction of SF2/ASFΔRS with U170K. HeLa cells were transfected with EGFP-SF2/ASFΔRS and cotransfected with either mCherry-C1 or mCherry-U170K. Confocal image of transfected cells and FLIM image of same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP- SF2/ASFΔRS + mCherry-C1 (Bottom) EGFP- SF2/ASFΔRS + mCherry-U170K **(B) FRET measured by FLIM for interaction of U2AF35 with SF2/ASFΔRS.** Experiments were performed as described in panel (A) except cells were cotransfected with EGFP-SF2/ASF and mCherry-SF2/ASF.

Based on *in vitro* data the ability of SF2/ASFΔRS to interact with U170K and U2AF35 is unexpected. The presence of RNA and endogenous proteins within the cell may stabilise the complexes containing SF2/ASFΔRS to such an extent that FRET is observed.

Following the observation that deletion of the RS domain of SF2/ASF did not abolish the interactions with U170K and U2AF35 in live cells, it was investigated whether point mutations in the second RRM (RRM2) of SF2/ASF would perturb these interactions. The mutant, WQD-AAA (herein referred to as SF2/ASF AAA) was generated by Giuseppe Biamonti's lab (Chiodi et al., 2004) by replacing three surface residues in the heptapeptide, SWQDLKD, that composes the first α-helix of RRM2 and is conserved in all the SR proteins containing an atypical RRM (Birney et al., 1993). According to the RRM model, this extended α-helix is not involved in RNA binding but has a structural function and could be available for protein-protein interactions (Dauksaite and Akusjarvi, 2002; Ge et al., 1998; Petersen-Mahrt et al., 1999). Mutations in this heptapeptide have been shown to affect the function of SF2/ASF in translation (Sanford et al., 2005) and alternative splicing (Chiodi et al., 2004).

I have tested the ability of SF2/ASF AAA to interact with U170K and U2AF35 by acceptor photobleaching. Figure 3.24(A) shows that point mutations in the second RRM are not sufficient to abolish the interaction with U170K or U2AF35 in live cells. Furthermore, U170K was shown to be capable of interacting with SF2/ASF AAA in Co-IP experiments (Figure 3.24(B)). This interaction appears to be reduced by treatment with RNase (compare lanes 4 and 5).

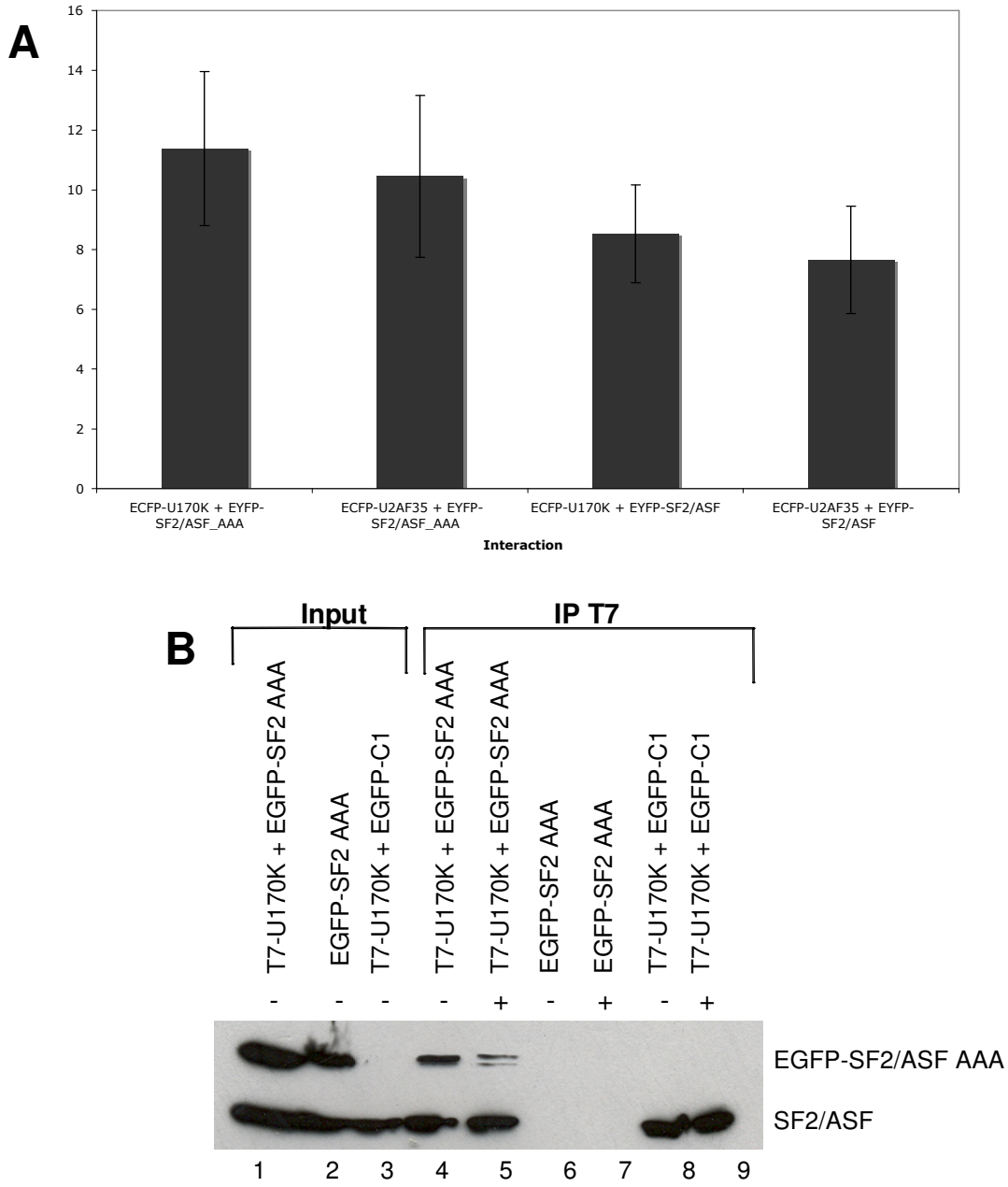


Figure 3.26: (A) FRET efficiencies for interaction of SF2/ASF AAA with U170K and U2AF35. Plot of FRET efficiencies (average for 7 to 9 cells) between CFP + YFP pairs measured by FRET acceptor photobleaching. Mean +/- SD. **(B) EGFP-SF2AAA interacts with T7-U170K in cultured mammalian cells.** Extracts prepared from 293T cells either transiently transfected with EGFP-SF2/ASF AAA and pCG-T7-U170K (lanes 4 and 5) EGFP-C1 and pCG-T7-U170K (lanes 8 and 9) or EGFP-SF2/ASF AAA (lanes 6 and 7) were incubated with anti-T7 antibody bound to sepharose beads. The bound proteins were analysed by Western blotting with anti-SF2 antibody. Alternatively the immunoprecipitate was treated with RNAase (lanes 5, 7 and 9). Lanes 1,2 and 3 were loaded with 2% of the amount of extract used for each IP.

3.16 HCC1 interacts with both U2AF35 and U2AF65

A gene-trap screen carried out in our laboratory designed to identify novel proteins located in nuclear speckels isolated a novel SR-related protein, SRrp53, required for the second step of splicing (Cazalla et al., 2005). Yeast two-hybrid and Co-IP experiments identified HCC1 as interacting partner of SRp53 (Cazalla et al., 2005). HCC1 is highly related to U2AF65 (Imai et al., 1993), although a role in constitutive splicing is unclear (Dowhan et al., 2005). It has been proposed that HCC1 interacts with components at the 3' splice site possibly replacing U2AF65 at the polypyrimidine tract and forming a U2AF-like complex with U2AF35. The formation of this complex may be facilitated by interactions with SRrp53 (Cazalla et al., 2005). Moreover, several factors related to U2AF35 have been characterized in mammalian cells suggesting the existence of multiple U2AF-like complexes (Tronchere et al., 1997; Shepard et al., 2002).

I have characterised the protein interaction partners of HCC1 by Co-IP experiments and subsequently by FRET microscopy. Figure 3.25(A) shows EGFP-U2AF35 is capable of pulling down T7-epitope-tagged HCC1.4 in Co-IP assays carried out in 293T cells. The hypothesis that HCC1 may form an alternative U2AF-like complex with U2AF35 was challenged by the observation that endogenous HCC1 can pull down both U2AF35 and U2AF65 in Co-IP assays (Figure 3.25). This data suggests that HCC1 interacts with the U2AF heterodimer in an RNA-independent manner, although it is possible that HCC1-U2AF35 and HCC1-U2AF65 complexes exist within the cell.

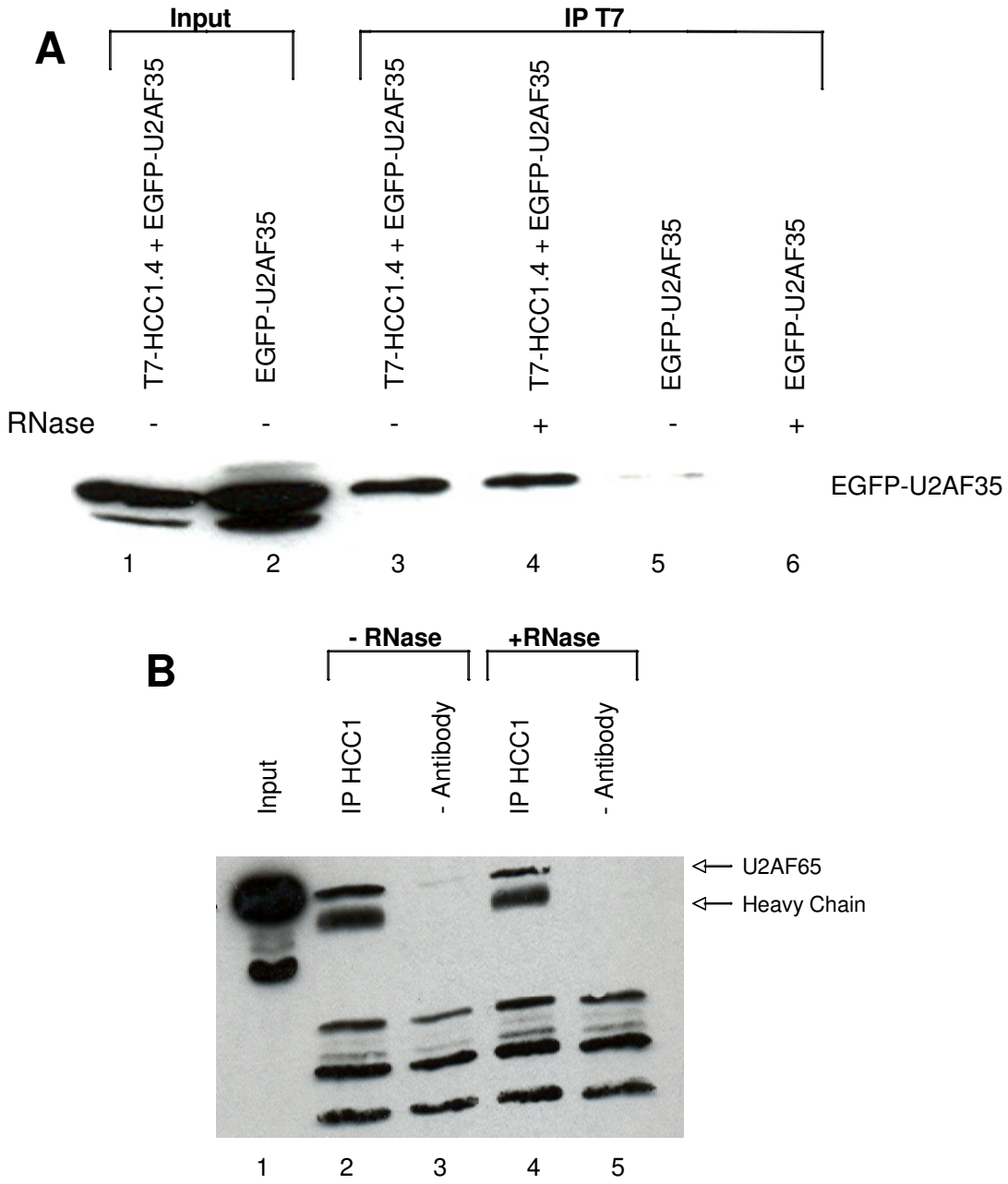


Figure 3.27: (A) EGFP-U2AF35 interacts with T7-HCC1.4 in cultured mammalian cells. IP assays with T7-HCC1.4. Extracts prepared from 293T cells either transiently transfected with EGFP-U2AF35 and pCG-T7-HCC1.4 (lanes 3 and 4) or EGFP-U2AF35 (lanes 5 and 6) were incubated with anti-T7 antibody bound to sepharose beads. The bound proteins analysed by Western blotting with anti-GFP antibody. Alternatively the immunoprecipitate was treated with RNAase (lanes 4 and 6). Lanes 1 and 2 were loaded with 2% of the amount of extract used for each IP. **(B) U2AF65 interacts with HCC1 in cultured mammalian cells.** IP assays with endogenous HCC1. Extracts prepared from 293T were incubated with either anti-HCC1 antibody bound to sepharose beads (lanes 2 and 4) or sepharose beads alone (lanes 3 and 5). The bound proteins were analysed by Western blotting with anti-U2AF65 antibody. Alternatively the immunoprecipitate was treated with RNAase (lanes 4 and 5). Lane 1 was loaded with 2% of the amount of extract used for each IP.

I have further characterised the protein-protein interactions of HCC1 by acceptor photobleaching. Cotransfection of ECFP-HCC1 and either EYFP-U2AF35 or EYFP-U2AF65 resulted in significant FRET being observed that was not significantly altered upon DRB treatment (Figure 3.26(A)). Importantly as previously demonstrated by acceptor photobleaching and *in vitro* assays I can confirm U2AF65 does not interact with itself by acceptor photobleaching (Chusainow et al., 2005).

The subnuclear localisation of protein-protein interactions between EGFP-HCC1 and mCherry-U2AF35, and mCherry-U2AF65 has been mapped by FLIM microscopy. The highest regions of FRET for the interaction of EGFP-HCC1 with mCherry-U2AF35 were not confined to the speckles as was previously observed for the interactions involving SF2/ASF, instead there appears to be variations between individual speckles within the cell (Figure 3.27). For the interaction of EGFP-HCC1 with mCherry-U2AF65 it is very clear that the highest regions of FRET occur within discrete domains within the nucleoplasm and not within the speckles (Figure 3.28). Similar results have been observed with ECFP-HCC1 and EYFP-U2AF65 by FLIM microscopy (data not shown). Figure 3.28 (lower panel) shows that these regions of high FRET persist in the nucleoplasm upon treatment with DRB. A t-test has been applied to demonstrate that a significant increase in FRET efficiency for the interaction of HCC1 with U2AF35 or U2AF65 is observed upon treatment with DRB. This may reflect the formation of complexes that are not disassembled upon inhibition of transcription. Further analysis will be required to check that the level of the acceptor fluorophore is equal in treated and untreated samples as the ratio of donor to acceptor has been shown to affect FRET efficiencies. Figure 3.26 shows that significant FRET is not observed between EGFP-U2AF65 and mCherry-U2AF65.

Therefore I have confirmed by both acceptor photobleaching and FLIM that this interaction does not result in significant FRET

Taken together these results show that HCC1 can interact with both subunits of the U2AF heterodimer and demonstrate that different complexes of splicing factors are distributed in different regions within the nucleus.

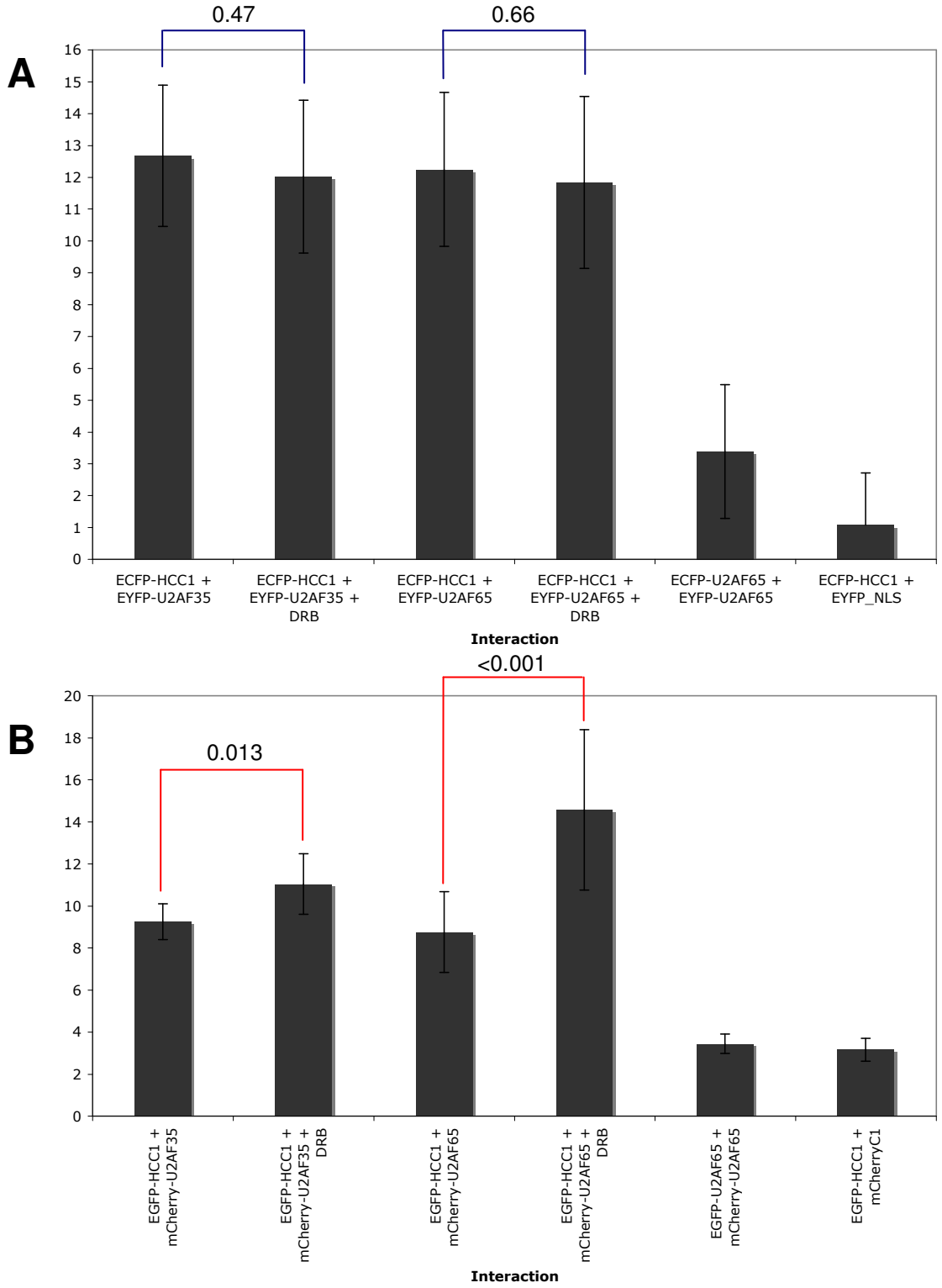


Figure 3.28: (A) Effect of DRB on interactions of HCC1 with U2AF35 and U2AF65. Plot of FRET efficiencies (average for 8 to 18 cells) between ECFP + EYFP pairs measured by FRET acceptor photobleaching. Mean +/- SD. **(B) FRET efficiencies determined by FLIM for interaction of HCC1 with U2AF35 and U2AF65 in the presence and absence of DRB.** FRET efficiencies between the EGFP and mCherry tagged pairs were determined by fixing the t2 to the mean lifetime for the donor cotransfected with mCherry-C1. Plot of mean FRET efficiencies +/- SD for 6 to 12 cells.

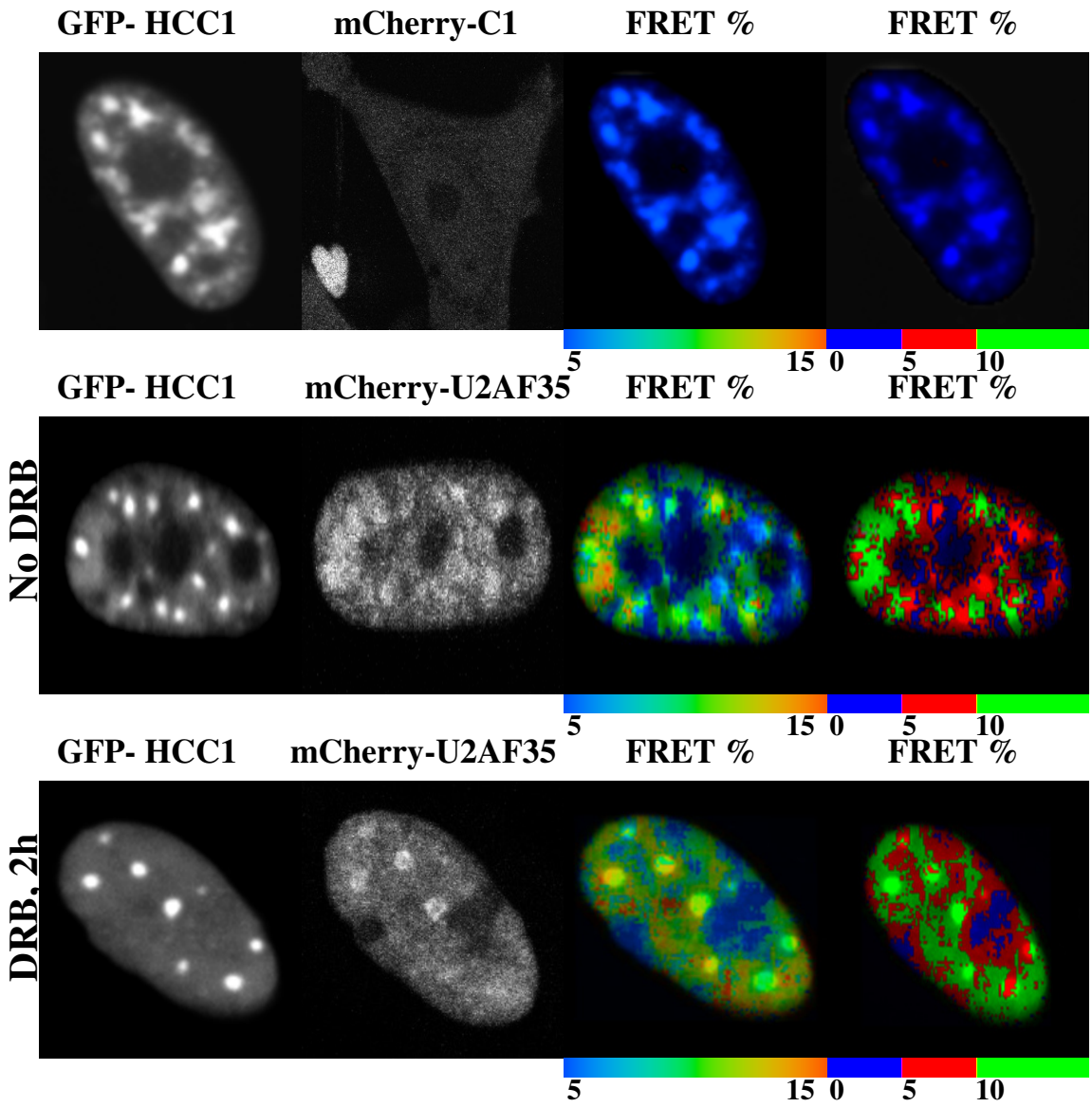


Figure 3.29: FRET between HCC1 and U2AF35 measured by FLIM. HeLa cells were transfected with EGFP-HCC1 and cotransfected with either mCherry-C1 or mCherry-U2AF35. Confocal image of transfected cells and FLIM image of same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies were calculated by fixing the t2 lifetime to the average lifetime value for EGFP-HCC1 cotransfected with mCherry-C1. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP-HCC1 + mCherry-C1 (Middle) EGFP-HCC1 + U2AF35-mCherry (Bottom) EGFP-HCC1 + U2AF35-mCherry, cells treated with 25 µg/ml DRB for two hours before imaging

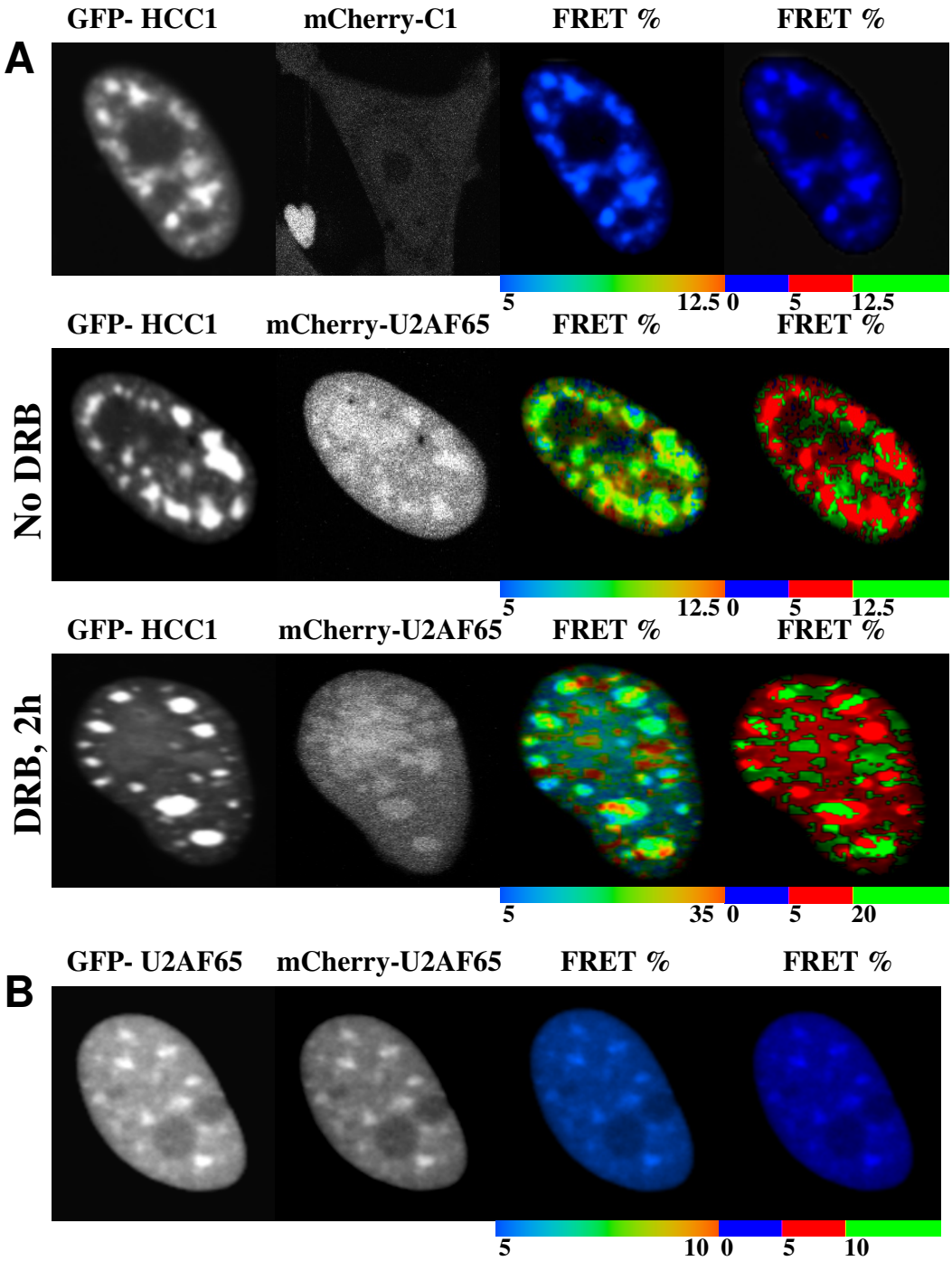


Figure 3.31: (A) FRET between HCC1 and U2AF65 measured by FLIM. HeLa cells were transfected with EGFP-HCC1 and cotransfected with either mCherry-C1 or mCherry-U2AF65. Confocal image of transfected cells and FLIM image of same cells, in which FRET Efficiency is shown in false colour. The colour scale with the respective efficiency (%) is indicated. The FRET efficiencies are shown in either continuous or discrete false colour. (Top) EGFP-HCC1 + mCherry-C1 (Middle) EGFP-HCC1 + U2AF65-mCherry (Bottom) EGFP-HCC1 + U2AF65-mCherry, cells treated with 25 µg/ml DRB for two hours before imaging. **(B) Non-significant FRET is observed between EGFP-U170K and mCherry-U2AF35.** HeLa cells were transfected with EGFP-U2AF65 and cotransfected with mCherry-U2AF65. Cells were analysed exactly as described for panel (A)

Discussion

Protein-protein interactions involved in spliceosome assembly have been studied extensively in the past using *in vitro* techniques and yeast two-hybrid analysis. However, the advent of FRET microscopy has made it possible to study the interactions between splicing factors in real-time in live cells, without perturbing the highly structured dynamic nature of the nucleus or introducing artificial salt concentrations. The importance of studying protein-protein interactions in live cells is demonstrated by previous work that shows that the association of the RNA-binding protein HuR with its target mRNA, c-fos, as detected by co-immunoprecipitatation, results largely from the reassociation of molecules subsequent to cell lysis (Mili and Steitz, 2004). The existence of such post-lysis rearrangements thus demonstrates that co-immunoprecipitation does not always recapitulate the *in vivo* state of ribonucleoprotein complexes.

The role of different subnuclear compartments within the nucleus has been investigated by studying the colocalisation of factors with a variety of nuclear bodies. However, by FRET microscopy it is possible to distinguish between those factors that simply reside in the same compartments and those that functionally associate with each other. Furthermore, biochemical isolation of nuclear bodies only captures their composition at a particular moment in time and does not allow the rapid trafficking of molecules to and from nuclear bodies to be studied (reviewed by (Dundr and Misteli, 2001b)). To further investigate the function of nuclear bodies, assays capable of localising specific molecular complexes are needed. To this end, I have used FRET microscopy to study a variety of splicing factor complexes. Firstly, FRET acceptor photobleaching was used to confirm that the fluorescently tagged proteins can interact

within a localised area within the cell and subsequently FLIM microscopy was employed to precisely map the distribution of splicing complexes throughout the cell. The existence of splicing complexes previously isolated *in vitro* has been confirmed in living cells and the technique has been used to identify novel splicing complexes.

4.1 The protein-protein interactions involved in exon and intron definition occur in living HeLa cells

Because the detection of FRET between two fluorescent molecules depends strongly on their proximity (Patterson et al., 2000), this technique can provide strong evidence that two proteins interact within a cell. However FRET alone cannot confirm a direct protein-protein interaction. Additional components, that may be proteins or RNA, may be required to stabilise the complexes containing the fluorescently tagged proteins. Under these circumstances, FRET would be observed as long as the donor and acceptor fluorophores are in an optimal orientation and are brought to within 1 and 10 nm of each other. Therefore, initially I used FRET microscopy to study the interactions between splicing factors that had already been shown to directly interact with each other *in vitro*.

The SR proteins, SF2/ASF and SC35, have previously been shown to interact with U170K and with U2AF35 by *in vitro* approaches (Wu and Maniatis, 1993; Kohtz et al., 1994). Furthermore, yeast two-hybrid analysis has demonstrated that SR proteins can interact simultaneously with U170K and U2AF35 (Wu and Maniatis, 1993). Therefore it was proposed that SR proteins play a role in exon and intron definition by interacting with U170K bound at the 5' splice site and U2AF35 bound at

the 3' splice site. I have used FRET microscopy to demonstrate that the SR proteins SF2/ASF and SC35 interact with both U170K and U2AF35. I have also extended the original *in vitro* studies to show that another SR protein family member, SRp20, interacts with U170K and U2AF35.

The ability of each individual SR protein to complement an inactive S100 cytosolic extract suggests SR proteins have redundant function in the constitutive splicing of certain introns. However, several differences in the ability of these proteins to regulate alternative splicing suggested that individual SR proteins have unique functions in splicing regulation (Caceres et al., 1994; Wang and Manley, 1995; Chandler et al., 1997). In addition genetic analyses of SR proteins demonstrates that particular SR proteins have specific functions in certain tissues or at particular developmental stages (Wang et al., 1998b; Jumaa et al., 1999; Wang et al., 2001; Xu et al., 2005).

The mechanism of splice-site selection in alternative and constitutive splicing are closely related (Horowitz and Krainer, 1994) therefore the ability of numerous SR proteins to interact with U170K and U2AF35 may be important for their role in regulating alternative splicing. At this point in time, SRp54 is the only SR protein that does not interact with U170K and U2AF35 but instead interacts with U2AF65 (Zhang and Wu, 1996). However, a role for SRp54 in the intron bridging of small *Drosophila* introns has been suggested (Kennedy et al., 1998).

I have demonstrated that SR proteins interact with U170K and U2AF35 with a similar FRET efficiency and that the protein-protein interactions between SR proteins and U170K or U2AF35 have a similar distribution within the nucleus. However, it has not been demonstrated by FRET microscopy that SR proteins interact simultaneously with U170K and U2AF35. This could be resolved by carrying out

three-chromophore FRET analysis that allows multiprotein complexes to be studied in living cells (Galperin et al., 2004).

4.2 The interactions between splicing factors are not exclusively cotranscriptional

The protein-protein interactions between U2AF35 and U2AF65 have previously been studied by FRET acceptor photobleaching in the presence and absence of the pol II inhibitor, DRB, to analyse the effect upon this interaction of inhibiting transcription and therefore splicing (Chusainow et al., 2005). Upon treatment with DRB no significant decrease in the FRET efficiency for this interaction was observed. I have studied numerous interactions between splicing factors and have shown that none of them are abolished by treatment with DRB. This data suggests that certain splicing factors form complexes within the cell before being recruited cotranscriptionally to the spliceosome.

Further evidence that SR proteins and U170K associate with each other before they are recruited cotranscriptionally to the spliceosome has come from studies of factors associated with pol II. A comprehensive proteomic analysis of immunopurified human pol II identified over 100 specifically associated proteins (Das et al., 2007). Among these are the SR proteins and all the components of the U1 snRNP, but no other snRNP or splicing factors. This has led to a model being proposed whereby the association of U1 snRNP and SR proteins with pol II results in their cotranscriptional recruitment to nascent transcripts to promote spliceosome assembly. Further evidence for this model is provided by the observation that splicing

efficiency is strongly enhanced if SR proteins are available during transcription but not if they are added immediately after transcription. FRET microscopy could be employed to study the association of splicing factors with pol II. It would be particularly interesting to determine whether the association of splicing factors with pol II requires ongoing transcription.

Chromatin immunoprecipitation assays that detect transcription-dependent accumulation of splicing factors have demonstrated that splicing factors are poorly detected on intronless genes, a result that opposes direct recruitment by Pol II or the cap binding complex *in vivo* (Gornemann et al., 2005; Listerman et al., 2006).

Currently only components of the U1 and U2 snRNP (or non-snRNP-associated splicing factors) have been studied using FRET microscopy. Studying the protein-protein interaction of U4, U5 or U6 associated proteins may enable us to determine whether the snRNPs engage the pre-mRNA as a penta-snRNP (Stevens et al., 2002) or whether they are recruited sequentially. For example if the snRNPs are recruited sequentially FRET between U2 and U5 associated proteins would be expected to occur only after formation of the spliceosomal B complex and therefore should not occur in transcriptionally inactive cells.

I was able to confirm that SR proteins interact with U170K and U2AF35. However, due to the fact that these interactions do not appear to occur exclusively in the spliceosome it has not been possible to determine whether these interactions occur on the pre-mRNA or whether they play a role in exon and intron definition. To address the question of whether FRET can be detected between splicing factors interacting on a pre-mRNA the MS2-GFP system, that allows transcription to be visualised in live cells, could be employed (Janicki et al., 2004). It has previously been demonstrated that induction of a highly expressed gene leads to the recruitment

of splicing factors to the site of transcription (Misteli et al., 1997), this would be expected to be accompanied by an increased FRET efficiency at the site of transcription. Using a variety of reporters with mutated binding sites or variable lengths of exons or introns could lead to key insights into whether SR proteins play a role in bridging exons and introns in live cells.

4.3 The role of the RS domain in pre-mRNA splicing

The role of the RS domain in pre-mRNA splicing in living cells was investigated by studying the protein-protein interactions of a mutant of SF2/ASF that lacks the C-terminal RS domain (SF2/ASFΔRS) with U170K and U2AF35 using FRET microscopy. The traditional view that SR and SR-related proteins contact the pre-mRNA through their RRMs and that the RS domain acts as a protein-protein interaction domain is now being challenged by studies that show that U2AF65 and SR proteins contact the pre-mRNA through their RS domains at several stages during spliceosome assembly (Valcarcel et al., 1996; Shen et al., 2004; Shen and Green, 2004; Hertel and Graveley, 2005). While some experiments have shown that SR proteins bound to an enhancer promote the binding of U2AF to the 3' splice site of a regulated intron (Wang et al., 1995; Graveley et al., 2001), through the RS domain of U2AF35 (Zuo and Maniatis, 1996), other experiments experiments failed to observe changes in U2AF recruitment in the presence or absence of an enhancer (Li and Blencowe, 1999; Kan and Green, 1999). Genetic experiments in *Drosophila* have demonstrated that flies expressing a version of dU2AF38 (the homolog of U2AF35) that lacks its RS domain – which, therefore should not interact with SR proteins – do not display any defects in the splicing of doublesex pre-mRNA, one of the model

genes for which U2AF recruitment was shown *in vitro* (Rudner et al., 1998). Furthermore, in contrast to the essential role of U2 snRNP recruitment *in vitro*, the RS domain of the Drosophila large subunit homologue (dU2AF50) was dispensable *in vivo* (Rudner et al., 1998). Therefore in contrast to the separate roles assigned to the U2AF RS domains *in vitro*, they may have redundant functions *in vivo*.

The RS domain of SF2/ASF is dispensable for the concentration-dependent effects on alternative splice-site-selection and for splicing of several substrates, including constitutive and enhancer-dependent pre-mRNAs (Caceres and Krainer, 1993; Zhu and Krainer, 2000). Furthermore, the RS domain of SF2/ASF was found not to be required for enhancing U1 snRNP binding to alternative 5' splice sites (Eperon et al., 2000) and it is not sufficient for the interaction with U170K (Xiao and Manley, 1997). Therefore SR proteins have an RS domain-independent function in constitutive and enhancer-dependent splicing that may include regulating protein-protein interactions through their RRMs (Ge et al., 1998) or competing with negative factors, such as hnRNPs, for the nascent pre-mRNA. It has previously been shown that SF2/ASF lacking an RS domain can displace hnRNP A1 from the pre-mRNA (Eperon et al., 2000). Furthermore, the identity of the RS domain is not important in *in vivo* alternative splicing assays, as RS domains are functionally interchangeable. In contrast RRM2 of SF2/ASF has a dominant role and can confer specificity to a heterologous protein (van der Houven van Oordt et al., 2000).

I have demonstrated that both U2AF35 and U170K can interact with SF2/ASF lacking an RS domain (SF2/ASFΔRS) by FRET microscopy. This suggests that other domains of SF2/ASF may have roles in mediating protein-protein interactions *in vivo*. The observation that SF2/ASF can interact with other SR proteins (Wu and Maniatis, 1993) and with itself indicates that SF2/ASFΔRS may be incorporated into a complex

with the wild type endogenous SR proteins and the fluorescently tagged U170K or U2AF35 resulting in significant FRET being observed. The presence of RNA in the cell may also be important for stabilising this complex. The fact that SF2/ASFΔRS can function in the splicing of certain substrates (Zhu and Krainer, 2000) and that significant FRET is observed in the nucleoplasm, which could correspond to active sites of splicing, suggests that SF2/ASFΔRS can be incorporated into the spliceosome.

The fact that SR proteins can self-interact provides (Wu and Maniatis, 1993) an explanation as to how SR proteins can be involved in contacting the pre-mRNA through their RS domain and simultaneously interact with both U170K and U2AF35. It could be proposed that for each molecule of U170K and U2AF35 there are multiple SR proteins leaving some RS domains free to contact the pre-mRNA (Figure 4.1).

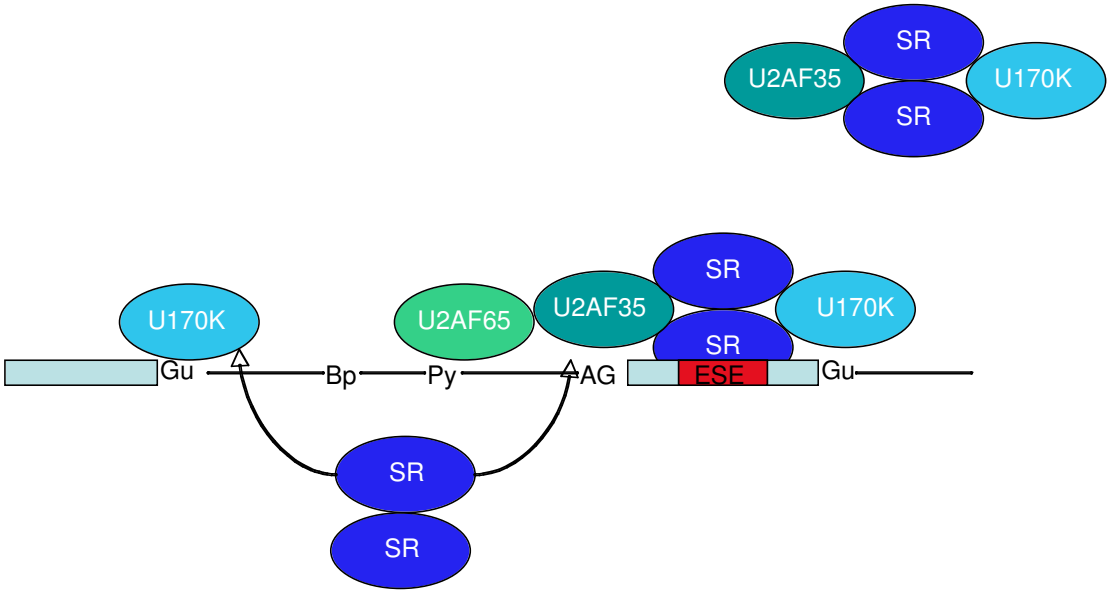


Figure 4.1 Proposed model for the interactions of SR proteins with U170K and SF2/ASF. SR proteins have been observed to self-interact leading to the proposal that for each molecule of U170K and U2AF35 there are multiple SR proteins. The U170K-SR protein-U2AF35 complex is proposed to form independent prior to it's recruitment to the spliceosome.

4.4 HCC1 interacts with both subunits of the U2AF heterodimer

The role of HCC1, a factor highly related to U2AF65, in constitutive splicing is poorly understood. An alternatively spliced isoform of HCC1, HCC1.3 (also called CAPERα), was purified as a spliceosome component capable of affecting the splicing reaction (Jung et al., 2002; Hartmuth et al., 2002; Rappsilber et al., 2002; Auboeuf et al., 2004) and has been shown to regulate transcription and alternative splicing in a steroid hormone-dependent manner (Dowhan et al., 2005). Previously it has been shown that an alternative isoform of HCC1 containing an additional 6 amino acids, HCC1.4, interacts with an SR-related protein, SRrp53 that can activate weak 3' splice sites (Cazalla et al., 2005). Furthermore, a second U2AF65-like component, PUF60, has been identified that binds to the polypyrimidine tract and regulates the alternative splicing of a subset of exons (Page-McCaw et al., 1999; Hastings et al., 2007). Mass spec analysis of PUF60 protein interaction partners identified U2AF65 and HCC1 as well as SR proteins and components of the U1 snRNP (Hastings et al., 2007). Finally, U2AF65, PUF60 and HCC1 have all been shown to interact with SRp54 which has been implicated in early 3' splice site recognition (Zhang and Wu, 1996; Page-McCaw et al., 1999; Dowhan et al., 2005). This has led to the proposal that U2AF65-like factors can interact with components required for the early recognition of the 3' splice site and influence the commitment to splicing.

Due to the high degree of homology between HCC1 and U2AF65 it could be proposed that HCC1 can bind to the polypyrimidine tract in place of U2AF65 and interact with a U2AF35 to form a U2AF-like complex (Figure 4.2(A)). However, FRET microscopy has demonstrated that HCC1 is capable of interacting with both

subunits of the U2AF heterodimer this has led to the proposal of two alternative models. The ability of U2AF35 but not U2AF65 to self-interact (Chusainow et al., 2005) may facilitate the formation of a complex containing HCC1 and both subunits of the U2AF heterodimer (Figure 4.2 (B)). This model assumes that the self-interaction of U2AF35 does not prevent the interaction with U2AF65. At this point in time the domain responsible for the self-interaction of U2AF35 has not been mapped. In the second model HCC1 forms binary complexes with either U2AF35 or U2AF65 (Figure 4.2(C). However, it remains possible that both situations could exist within the cell

There are several ways to distinguish between these two models. It would be interesting to carry out a FRET analysis with various mutants of HCC1 to determine the domains responsible for the interactions with U2AF35 and U2AF65. If the model presented in Figure 4.2 (B) is correct it is more likely that different domains of HCC1 mediate the interactions with U2AF35 and U2AF65. It would also be interesting to determine whether mutations affecting the self-interaction of U2AF35 affect the interaction of HCC1 with the U2AF heterodimer.

Several biochemical approaches could be applied to distinguish between these two models. Gel filtration assays could be utilised to determine the size of the complex in which HCC1 resides. GST-pull down assays using recombinant proteins could be applied to determine whether the interaction of HCC1 with the U2AF heterodimer requires the presence of both subunits. If HCC1 is able to interact with U2AF65 in the absence of U2AF35 it would favour the model presented in Figure 4.2 (C).

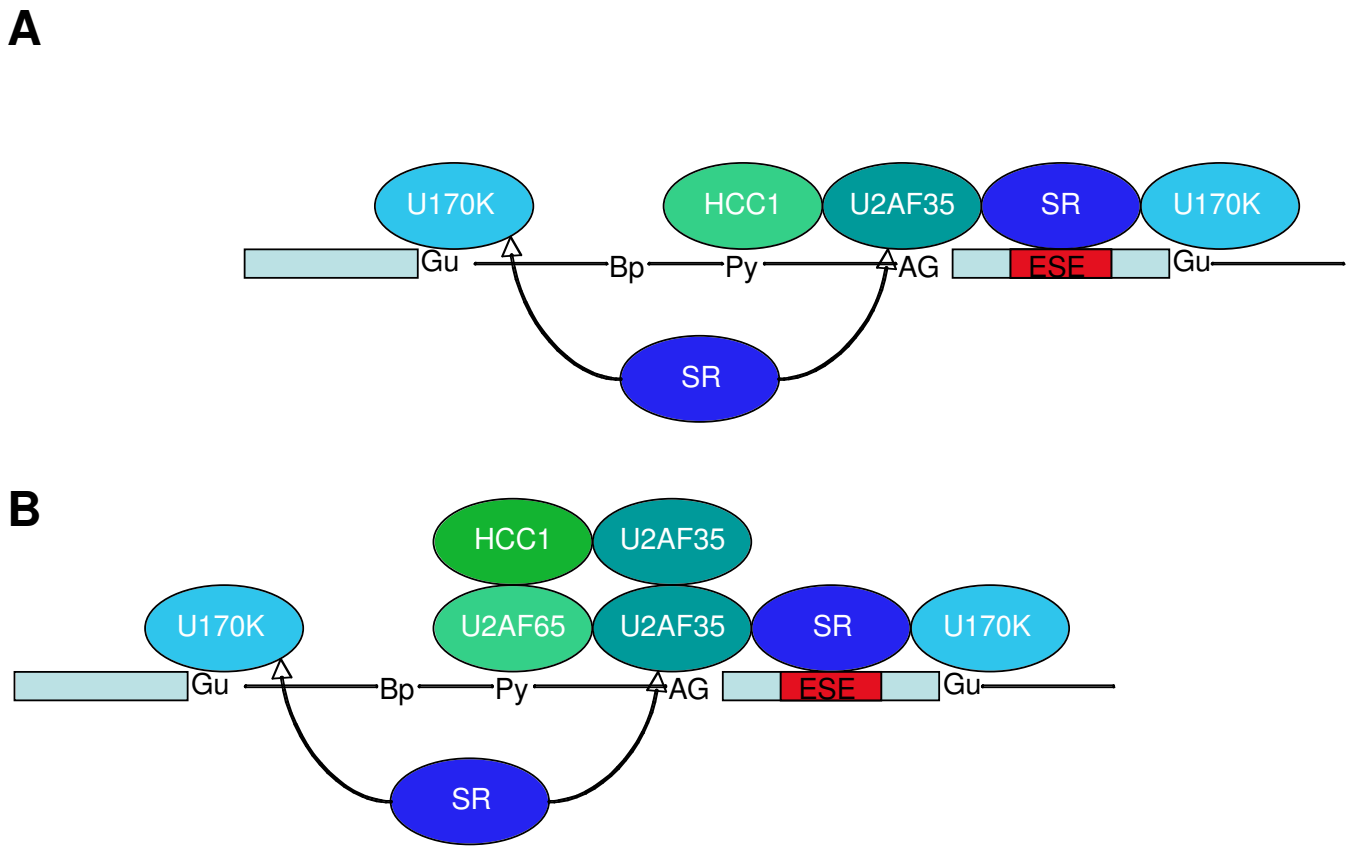


Figure 4.2: Proposed models for the role of HCC1 in splicing. (A) Based on the homology between HCC1 and U2AF65 it was proposed that HCC1 replaces U2AF65 at the polypyrimidine tract. **(B)** FRET analysis shows that U2AF35 can self-interact and HCC1 interacts with both subunits of the U2AF hetordimer. This has led to an alterntive model whereby the self-interaction of U2AF35 mediates the formation of a

complex containing HCC1 and U2AF65. **(C)** HCC1 forms binary complexes with both U2AF35 and U2AF65. These complexes may or may not exists on the pre-mRNA. The models presented in (B) and (C) may not be mutally exclusive.

There are several other approaches that could be taken to gain an insight into a potential role for HCC1 in constitutive splicing. While HCC1 has been shown to be present in the human spliceosome (Rappsilber et al., 2002) it has not been determined at which stage it is recruited or whether it interacts with the polypyrimidine tract. It would be interesting to determine the RNA-binding targets of HCC1 to determine whether it is recruited to a subset of transcripts. Finally it would be interesting to determine whether HCC1 is able to compensate for depletion of U2AF65.

Previously, a U2AF35-related protein (Urp) has been isolated and despite its homology to U2AF35 their functions do not overlap (Tronchere et al., 1997). Urp interacts with U2AF65 through a U2AF35 homologous region and with SR proteins through its RS domain, however co-immunodepletion showed that Urp is associated with the U2AF heterodimer and does not form an alternative U2AF-like complex with U2AF65. It has been proposed that Urp and U2AF35 independently position RS-domain-containing factors within spliceosomes. The observation that HCC1 can interact with the U2AF heterodimer and with SR-related proteins suggests a similar role for this protein in spliceosome assembly. The interactions of Urp and HCC1 with the U2AF heterodimer and the observation that U2AF35 can interact with itself (Chusainow et al., 2005) offers an insight into how U2AF35 can mediate interactions with other RS-domain-containing factors bound at the 5' splice site, assembled in splicing enhancer complexes, or associated with the U4/U6.U5 snRNP.

A second U2AF35-like factor, U2AF26, has been identified (Shepard et al., 2002). The N-terminal amino-acids of U2AF35 and U2AF26 are almost identical. However, the C-terminal domain of U2AF26 lacks many characteristics of the U2AF35 RS domain. U2AF26 can associate with U2AF65 and can functionally

substitute for U2AF35 in both constitutive and enhancer-dependent splicing, despite the absence of an RS domain. Therefore distinct U2AF-like complexes can function in pre-mRNA splicing. Further investigation will be required to determine whether HCC1 interacts directly with U2AF26 or Urp.

4.4 Subcellular distribution of splicing complexes

The subcellular distribution of protein-protein interactions has been mapped at nanometer resolution using FLIM. Previously fluorescent recovery after photobleaching (FRAP) analysis has shown that splicing factors are highly dynamic and shuttle rapidly between speckles and the nucleoplasm (Phair and Misteli, 2000; Kruhlak et al., 2000). Therefore these highly dynamic abundant factors may be constantly associating and disassociating with each other within the nucleus. Interestingly, we found that these protein-protein interactions have a differential distribution within the nucleus; whereas interactions involving SF2/ASF localized preferentially to the nuclear speckles, those involving SC35 preferentially localized to the nucleoplasm.

The protein-protein interactions involving HCC1 also mapped to discrete domains within the nucleus. While the highest regions of FRET between HCC1 and U2AF35 were observed in some speckles but not others the regions of highest FRET between HCC1 and U2AF65 were restricted to discrete domains within the nucleoplasm and absent from the speckles. As these domains persisted in the presence of DRB they probably don't correspond to active sites of transcription. However it remains to be determined whether post-transcriptional splicing occurs in these regions.

In summary, FLIM data demonstrate that the formation of splicing factor complexes is not exclusively regulated by the abundance of individual components, as the highest FRET efficiencies did not always occur in regions where splicing factors concentrate.

4.5 Speckles act as storage or assembly sites for splicing factors

In certain cases the highest FRET efficiencies were observed in the speckles. Numerous pieces of evidence suggest that speckles are not active sites of splicing but act as storage or assembly sites for splicing factors. Therefore, this provides further evidence that factors involved in intron and exon definition associate together in a complex before being recruited cotranscriptionally to the spliceosome.

In this thesis I provide further evidence that speckles act as storage or assembly sites for splicing factors. By comparing the FRET efficiencies in the nucleoplasm and the speckles in the presence and absence of DRB for the interaction of SF2/ASF with U170K or U2AF35 it was observed that DRB treatment causes FRET to decrease in the nucleoplasm relative to untreated cells. This is probably due to the fact that treatment with DRB causes speckles to become enlarged and rounded up as splicing factors are no longer recruited to active sites of transcription and therefore are not incorporated into the spliceosome. The changes in FRET efficiency observed upon treatment with DRB are consistent with the idea that in transcriptionally inhibited cells there are less protein-protein interactions between splicing factors in spliceosomes but more in storage or assembly sites.

4.6 Phosphorylation regulates spliceosome assembly

It remains to be determined how the distribution of splicing factor complexes are regulated within the cell and how splicing factors locate and interact with their correct partners. In vitro experiments have shown that the phosphorylation status of splicing factors is important for regulating the assembly and disassembly of the spliceosome (Mermoud et al., 1992; 1994; Tazi et al., 1993; Cao et al., 1997). Furthermore, phosphorylation of SF2/ASF has been shown to increase its affinity for U170K (Xiao and Manley, 1997). In the future it would be interesting to determine whether modulating signalling pathways or kinase activity is important for regulating the interactions between splicing factors in live cells. Previously it has been shown to be possible to detect FRET between an EGFP-tagged protein and fluorescently labelled phosphorylation-site-specific antibody (Ng et al., 1999; Verveer et al., 2000b; Wouters and Bastiaens, 1999). This could be applied to study the protein-protein interactions of phosphorylated and unphosphorylated splicing factors.

4.7 The advantages of FRET microscopy

A key advantage of FLIM microscopy is that it provides quantitative information about the distance between fluorophores and the proportion of interacting fluorophores. This quantitative analysis can be carried out in single cells and even separate subnuclear compartments. Therefore the location and stoichiometry of molecular interactions can be compared between different cells and subnuclear compartments. Recent advances in single-cell live cell imaging have led to the realisation that nuclear constituents are constantly mobile, and that each molecule has

unique kinetics. Thus, cells stochastically vary, implying that population analysis may be misleading (reviewed by (Shav-Tal et al., 2006)). It is interesting to note that I observe considerable variations in the FRET efficiency for each interaction when the average FRET efficiency is measured for a population of cells. To accurately analyse the causes of these variations stable cell lines with constant ratios of donor and acceptor molecules will be required. Future areas of research could include studying how FRET efficiencies vary between different cells types and how they are regulated as the cell cycle progresses.

A major disadvantage of FRET microscopy is that it cannot be used to confirm a negative protein-protein interaction. False negatives may occur when the donor and acceptor fluorescent proteins are: (1) perturbing the proteins to which they are fused (2) in close proximity but in the wrong orientation for FRET to occur (3) too far away from each other even when their fusion partners are interacting. Therefore it may be useful to combine FRET microscopy with complimentary techniques for imaging the dynamics of protein interactions in living cells (reviewed by (Day and Schaufele, 2005)). Fluorescent recovery after photobleaching (FRAP) and fluorescent correlation spectroscopy (FCS) allow the dynamics of fluorescent-labelled molecules to be measured in living cells. Importantly, the interaction of proteins changes their kinetic properties therefore providing information about the presence of immobile populations that are stably bound to structures within the photobleached region, in the case of FRAP (Kruhlak et al., 2000), or allowing the assembly and disassembly of protein complexes to be studied.

Protein-protein interactions can also be imaged in living cells by bimolecular fluorescence complementation assays (Kerppola, 2006). However, FRET has several key advantages over protein complementation assays. FRET is instantaneous, fully

reversible (that is, it monitors disassociation as well as association) and has a well characterised dependence on distance and orientation. In contrast, protein complementation takes from minutes to hours for the fragments to fold, reversibility is absent or uncertain and the requirements on the conformation and affinity of the partner proteins are quantitatively ill-defined except that they must bring the two reporter fragments into the correct juxtaposition.

The well defined relationship between FRET efficiency and distance could lead to key insights into the structure of splicing complexes within living cells. Furthermore, intramolecular FRET, in which the donor and acceptor fluorophores are located in the same molecule, could be used to monitor conformational changes in splicing factors. It would be interesting to compare the structures analysed by FRET microscopy to those visualised by cryo-electron microscopy (Reed et al., 1988).

In summary, a platform is in place to study the components of the gene expression machinery in single live cells. FRET microscopy will allow the structure of gene expression factories to be determined and the location and stoichiometry of molecular interactions to be studied within the dynamic subcompartments of the nucleus. The system can easily be adapted to determine the effects of signalling pathways, the cell cycle, or the knockout of individual components on these molecular interactions.

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