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mRNA splicing in early mammalian embryos

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**A thesis submitted for the degree of Doctor of
Philosophy**

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Abstract

The overall aim of the experiments presented in this thesis is to investigate the onset and regulation of mRNA splicing in early mammalian development.

To investigate splicing efficiency in single mouse embryos, we have constructed splicing probes that exploit the properties of fluorescent proteins. A DsRed Express-intron-EGFP construct would report splicing by the expression of the fusion DsRed-EGFP protein, when injected in mouse embryos. In the absence of splicing the vector yields only DsRed. The ability of the probe to monitor splicing was confirmed by compromising the U1 small nuclear ribonucleoprotein or by mutation of the intron's 5' splice site. Furthermore, I show that inhibiting U1-mediated splicing at the onset of development inhibits development beyond the 2-cell stage.

Following the characterization of the splicing probe, the development of nuclear speckles was investigated during early mouse development by determining the nuclear localisation of the splicing factor SC35. These experiments showed a developmental change in nuclear organisation during oocyte maturation and through embryo development. We could also show that splicing takes place as early as the 1-cell stage embryos in the absence of nuclear speckles.

Finally, the role of an SR protein, SRp38, was also investigated. SRp38 has been reported to be a potent splicing inhibitor when dephosphorylated in mitotic cells or under heat stress. SRp38 was present during all stages of the early mouse development. Oocytes and embryos were found to be sensitive to the levels of SRp38. Expression of SRp38-EGFP or depletion of SRp38 using morpholinos caused an arrest of oocytes at the GV stage and embryos at the 2-cell stage. The

splicing reporter was used to reveal that these effects were independent of splicing suggesting a novel role for SRp38 in cell biology.

These studies provide a novel approach to monitoring splicing activity in single living cells and that splicing activity is necessary for the onset of early mammalian development.

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Abbreviations

| | |
|-----------|---|
| ATP | Adenosine Triphosphate |
| AU | Arbitrary Unit |
| BP | Band Pass |
| BSA | Bovine Serum Albumin |
| CMV | Cytomegalovirus |
| CTD | Carboxy-terminal domain |
| dsRNA | double-stranded RNA |
| dSRp38 | dephosphorylated SRp38 |
| EGA | Embryonic Genome Activation |
| EGFP | Enhanced Green Fluorescent Protein |
| ER | Endoplasmic Reticulum |
| ESE | Exonic Splicing Enhancer |
| ESS | Exonic Splicing Silencer |
| FITC | Fluorescein Isothiocyanate |
| FSH | Follicle Stimulating Hormone |
| GFP, EGFP | Green Fluorescent Protein (Enhanced) |
| GV | Germinal Vesicle |
| GVBD | Germinal Vesicle Breakdown |
| hCG | human Chorionic Gonadotrophin |
| hnRNP | heterogeneous nuclear ribonucleoprotein |
| IBMX | 3-isobutyl-1-methylxanthine |
| ICSI | Intra-Cytoplasmic Sperm Injection, |
| IGC | Interchromatin Granule Clusters |
| ISE | Intronic Splicing Enhancer |
| ISS | Intronic Splicing Silencer |
| IU | International Units |
| IVF | <i>In Vitro</i> Fertilisation |
| LH | Luteinising Hormone |
| LP | Long Pass |
| MGA | Mid-preimplantation gene activation |
| MI | Meiosis I |
| MII | Meiosis II |
| NEBD | Nuclear Envelope Breakdown |
| NES | Nuclear Export Signal |
| NLS | Nuclear Localisation Signal |
| NMD | Non-sense mediated decay |
| NSN | Non-surrounded nucleolus |
| PAP | PolyA polymerase |
| Pb | Polar body |

| | |
|------------------|--|
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PF | Perichromatin Fibrils |
| PIP ₂ | Phosphatidyl Inositol biphosphate |
| PKA | Protein Kinase A |
| PLC | Phospholipase C |
| PMSG | Pregnant Mare Serum Gonadotrophin |
| PN | Pronucleus |
| PTC | Premature termination codon |
| PVA | Polyvinyl Alcohol |
| RFP | DsRed Express |
| RNAi | RNA interference |
| RPB1 | RNA Polymerase II largest subunit |
| RRM | RNA Recognition Motif |
| RS | Serine/Arginine rich domain |
| RT | Room Temperature |
| SN | Surrounded Nucleolus |
| Sxl | Sex lethal gene |
| U2AF | U2 auxiliary factor |
| UsnRNP | small nuclear ribonucleoprotein particle |
| UTR | untranslated region |
| ZGA | Zygotic Genome activation |

1. General Introduction

In all eukaryotic organisms, gene expression requires splicing. Splicing is a process in which short intervening sequences that interrupt the coding sequence in a newly transcribed mRNA are excised to yield mRNA with the appropriate reading frame of a particular protein. Splicing is an essential step for gene expression in higher eukaryotes but it has never been studied during early development.

This thesis presents the design of a novel probe for monitoring splicing efficiency using fluorescent proteins, in single living embryos. Further experiments presented in this thesis have been designed to investigate two SR proteins with opposing properties during the early mouse development.

To introduce this topic I will review oogenesis and preimplantation development, followed by the mechanisms involved in gene expression and splicing.

1.1 Early mammalian development

1.1.1 Oocyte development

In mammals the population of oocytes that contribute to fertility are present at the time of birth. They are arrested in diplotene of the first meiotic division and are surrounded by a single layer of pre-granulosa cells. These are known as primordial follicles.

In response to an as yet unidentified signal, primordial follicles are stimulated to enter the growing phase. The oocyte increases in diameter from 15-20 μ m to more than 100 μ m in some species. In the mouse, the oocytes within primordial follicles have a diameter of 15 μ m (~0.9 pl in volume) and the fully grown oocyte, 80 μ m (~270 pl in volume). As the oocyte grows, the number of follicular granulosa cells increases in layers to form the primary follicle, secondary follicle and finally tertiary follicle with a large antral cavity (Chouinard, 1975).

Throughout follicular development the granulosa cells communicate with the oocyte via gap junctions (Anderson and Albertini, 1976; Kidder and Mhaw, 2002). These junctions are composed of connexins, a group of membrane proteins that allow the transport of small molecules (<1kDa) between adjacent cells (Herlands and Schultz, 1984). Connexins are essential for development of the oocytes past the preantral stage. This was shown in experiments where connexin 37 was knocked out in mice and development of the follicles was arrested (Carabatsos *et al.*, 2000). Furthermore, communication via oocyte secreted

factors is also necessary for oocyte development. An example is that of Growth Differentiation Factor-9 (GDF-9). Knock-out mice for GDF-9, are infertile due to failed ovarian follicular development (Carabatsos *et al.*, 1998). Finally another example of a secreted factor essential for follicular development is that of the kit-ligand (Packer *et al.*, 1994).

Concomitant with oocyte growth, a nuclear expansion also occurs. This enlarged nucleus is known as the germinal vesicle (GV). Changes in chromatin configuration also occur during oocyte development. These configurations have been termed “surrounded nucleolus” (SN) and “non surrounded nucleolus” (NSN) (Bouniol-Baly *et al.*, 1999). In the SN configuration, the condensed chromatin is arranged in a rim around the nucleolus of the germinal vesicle. However, in NSN the chromatin is not condensed and is distributed throughout the germinal vesicle and is not surrounding the nucleolus (Zuccotti *et al.*, 1995). Furthermore, it was shown that oocytes progress from the NSN configuration to the SN configuration approximately 17 days after birth (Zuccotti *et al.*, 1995). The percentage of SN oocytes was also shown to increase with the increase in oocyte size. Transcriptional activity has also been investigated in these two stages of oocyte development. NSN stage oocytes can actively transcribe, while SN stage oocytes do not (Bouniol-Baly *et al.*, 1999). This suggests that in SN stage oocytes, the genes required to support early development have been transcribed and stored for later translation. Finally, meiotic and developmental competence is significantly higher in oocytes that have progressed to the SN configuration at the appropriate

time, 15-17 days after birth. This suggests that the chromatin configuration is finely tuned with the control of expression and this is essential for further oocyte development (Zuccotti *et al.*, 1995; Bouniol-Baly *et al.*, 1999; Liu and Aoki, 2002).

1.1.2 Oocyte maturation

During oocyte growth the oocyte remains arrested in the diplotene stage of meiosis. Oocyte maturation refers to the progression from diplotene through metaphase I to arrest at metaphase II. Oocyte maturation is stimulated in fully grown oocytes within antral follicles in response to LH (Lunenfeld *et al.*, 1975). Maturation can also be stimulated in vitro by releasing the oocyte from the follicle into suitable culture media (Edwards *et al.*, 1969). Oocytes gradually acquire competence to resume meiosis. Firstly, oocytes are able to undergo GVBD at 60µm and the ability to progress to MII is achieved in oocytes of 67µm in diameter (Sorensen and Wassarman, 1976).

After GVBD, the oocyte progresses to metaphase I. The chromosomes become attached to the developing spindle and align on the metaphase plate. The spindle is first distinguishable after about 5-6 hours after meiotic resumption and is fully formed by 9-10 hours (Wassarman and Albertini, 1994). The first indication of an animal-vegetal axis occurs at the time the fully formed spindle (Zernicka-Goetz, 2002) migrates to the oocyte cortex (Verlhac *et al.*, 2000). During anaphase I the homologous chromosomes separate to opposite spindle poles. Telophase I completes the cell division marked by the extrusion of the first

polar body (Pb1), 10-12 hours after the LH surge (Wassarman and Albertini, 1994). Following the completion of Meiosis I, the oocyte enters Meiosis II and progresses to form the second meiotic spindle, bypassing the interphase stage. The oocyte remains arrested at metaphase of the second meiotic division (MII) until fertilisation takes place.

Regulation of translation plays an important role in oocyte maturation. The fate of transcribed mRNA during oocyte growth is tightly controlled and is coordinated by a well defined process. Certain mRNAs are translated during maturation, whereas others are stored for translation during later periods of development. Regulation of translation is achieved by polyadenylation, deadenylation and association of different factors on the mRNA that will regulate expression (Richter *et al.*, 1990). Early experiments showed that tissue plasminogen activator mRNA (tPA) was expressed during meiotic maturation following 3' end polyadenylation (Strickland *et al.*, 1988). In the same study, inhibition of translation was achieved by anti-sense RNA complementary to the 3' end of the tPA. The timing of translation is regulated by polyadenylation. mRNAs with longer polyadenylated (polyA) tails (~150 residues) are translated, whereas mRNAs with shorter polyA tails (<90 residues) may be stored for later translation (Bachvarova, 1992). Deadenylation of mRNA can inhibit expression and promote storage. Furthermore, masking factors on the mRNAs with shorter polyA tails can inhibit the binding of translation initiation factors on the capping machinery, therefore inhibiting their expression (Richter and Theurkauf, 2001). This

repression of gene expression occurs during GVBD. Expression of the stored mRNAs is later achieved by the release of the masking factors from the mRNA, due to their phosphorylation by MPF, which leads to polyadenylation and expression of the mRNA (Eichenlaub-Ritter and Peschke, 2002).

1.1.3 Fertilisation

Following sperm release into the female reproductive tract, it goes through a process of competence for fertilisation, called capacitation (Aitken *et al.*, 1996). This process occurs during the transport of the sperm to the ovulated oocyte. Thousands of sperm penetrate the cumulus cell mass which surrounds the MII arrested oocyte and bind to the zona pellucida protein glycoprotein ZP3 (Wassarman *et al.*, 2001). The binding of the sperm to the oocyte's ZP3, triggers the acrosome reaction. In this essential process the acrosome, a secretory vesicle in the sperm head, releases hydrolytic enzymes that allow zona penetration (Wassarman *et al.*, 2001). Fertilisation is initiated at the time of sperm fusing to the oocyte's plasma membrane. The block of further sperm penetration and therefore polyspermy is achieved by the event of cortical granule exocytosis that results in the release of hydrolytic enzymes from the oocyte to the sub-zonal (perivitelline) space. These enzymes alter the zona glycoprotein structure, inhibiting further sperm penetration (Wassarman *et al.*, 2001).

Fertilisation results in the resumption of meiosis, releasing the oocyte from the MII arrest, allowing the segregation of the sister chromatids and leading to the

extrusion of the second polar body (Pb2). The amount of cytoplasm extruded in the second polar body is very small, conserving the volume of the early embryo, in contrast with the male meiotic process where all four daughter cells receive an equal amount of cytoplasm. Second polar body extrusion in the mouse occurs approximately 90 minutes after sperm-oocyte fusion (Wassarman *et al.*, 2001).

1.1.4 Preimplantation embryonic development

Following fertilisation, the maternal and paternal pronuclei form around four hours after the sperm-egg fusion. This stage marks the transition from meiotic to mitotic cell divisions. In the mouse, the paternally derived pronucleus develops first and is larger than the female counterpart (Latham, 1999). The pronuclei then reposition at the centre of the cell, in such a way that they are positioned closely but still remain separate.

The first embryonic division occurs 17-20 hours after fertilisation (Howlett and Bolton, 1985; Yanagimachi, 1994). The second embryonic division then occurs at 46-54 hours and the embryo reached the 8-cell stage by 60 hours. Following the expression of adhesion molecules at the 8-cell stage, the embryonic cells, called blastomeres, become tightly packed forming a compacted morula (Fleming *et al.*, 2000). This marks the initiation of different embryonic lineages. The outer cells will become the trophectoderm and the inner cell mass the embryo proper. These lineages are evident by 96 hours after fertilisation when the blastocyst forms. During this period of embryo development, the embryo is

transported through the oviduct into the uterus. Finally, the embryo will hatch from the zona pellucida four to five days after fertilisation and implant in the uterine wall (Timings from Hogan et al., 1994).

1.2 Regulation of gene expression during early development

Gene expression during early development is regulated by inactivation of transcription and regulation of translation thereafter during oocyte maturation (section 1.1.2). Transcription remains inactivated through the early stages of development, until 2-cell stage in mouse, 4-8-cell stage in human, 8-16-cell stage in cattle and most domestic species (Kanka, 2003). The stage at which embryos become dependent on transcription has typically been demonstrated by sensitivity to an RNA Polymerase II inhibitor, such as α -amanitin. For example, mouse embryos will develop up to the 2-cell stage in the presence of α -amanitin. Embryos cultured from 1-cell stage in the presence of α -amanitin showed some protein synthesis when tested for radiolabeled methionine incorporation. When α -amanitin was introduced in the medium at the early 2-cell stage, the embryos arrested, with no methionine incorporation observed. This finding showed that methionine incorporation at the 1-cell stage was due to expression of maternal transcripts whereas 2-cell stage embryos expressed newly transcribed messages (Flach *et al.*, 1982).

Regulation of gene expression is achieved in growing oocytes by the inactivation of transcription and later by precise translational control of the

maternal stored mRNAs. Activation of transcription following fertilisation marks the beginning of the zygotic genome activation, a step which is essential for development.

1.2.1 Embryonic Genome Activation (EGA or ZGA)

Zygotic genome activation is a complex process that apart from activation of transcription includes other nuclear and cytoplasmic events that will ensure normal embryogenesis. Transcriptional activation occurs in most species in a stepwise manner, called minor and major genome activation.

In the mouse, there is a small burst of transcription in the second half of the one-cell stage marking the minor-zygotic genome activation (Latham *et al.*, 1992; Ram and Schultz, 1993). These early experiments showed expression of luciferase driven by an SV40 promoter in 1-cell embryos. It was later shown that both nuclei contribute to the transcriptional activity of the one-cell stage embryo, but the activity of the male pronucleus is 4-5 times greater compared to that of the female pronucleus (Aoki *et al.*, 1997). This was shown in experiments involving BrUTP incorporation in the mRNA in the two pronuclei. However, as well as newly synthesised transcripts, maternal transcripts are also essential for the transition to the 2-cell stage (Aoki *et al.*, 2003; Zeng *et al.*, 2004). The first proteins that have been shown to be synthesised from maternal mRNAs are transcription factors (Wang and Latham, 1997; Worrall and Schultz, 1997; Kaneko *et al.*, 1997). Microarray studies have recently reported in more detail the groups

of genes involved in these stages of the ZGA. The main group of proteins which are expressed in the 1-cell embryo involve protein metabolism and phosphorylation, cell cycle, transcription and DNA metabolism (Zeng *et al.*, 2004). Even though some of the maternal mRNAs are essential for this early stage of development, up to 90% of the oocyte RNAs are degraded by the 2-cell stage (Nothias *et al.*, 1995; Zeng *et al.*, 2004; Hamatani *et al.*, 2004b). It has been recently shown for the first time that splicing takes place from the one-cell stage (Zeng and Schultz, 2005). This was shown by microinjecting a reporter gene into the male pronucleus of 1-cell embryos, and identifying the spliced products by means of PCR. A similar result is also presented later in this thesis.

The major zygotic genome activation occurs in the mouse at the mid-late two-cell stage (Latham *et al.*, 1991; Nothias *et al.*, 1996). The level of transcription is approximately 60-70% higher compared to that of one-cell stage embryos (Aoki *et al.*, 1997). Microarray analysis reveals that the majority of new messages at the 2-cell stage are involved in transcription, mRNA processing, phosphate metabolism, regulation of cell cycle (Zeng *et al.*, 2004) and pyruvate metabolism (Hamatani *et al.*, 2004a).

A third wave of transcription named mid-preimplantation gene activation (MGA) has been recently described which involves the stages from 4-cell to blastocyst (Zeng *et al.*, 2004; Hamatani *et al.*, 2004a; Zeng and Schultz, 2005). The proteins expressed between the 4-cell stage and the blastocyst stage involve metabolism, protein and phospholipid biosynthesis ribosome biogenesis (Zeng *et*

al., 2004), glucose metabolism and genes involved in the differentiation of the embryo to the inner cell mass and trophectoderm (Hamatani *et al.*, 2004a).

These three distinct periods of transcription reflect the major transitions in early development. The changing profile of expressed mRNAs at each of those transitions is related to the major functions at each stage.

1.2.2 Regulation of transcription depends on RNA Polymerase II.

The state of the RNA Polymerase II plays an important role in the activation of the zygotic genome. In oocytes and the first few hours after fertilisation, the largest subunit of RNA Polymerase II (RPB1) is localised in the cytoplasm with its carboxy-terminal domain (CTD) hyperphosphorylated. The CTD becomes dephosphorylated several hours after fertilisation, at the same time that the first transcriptional activity is occurring, and by that time it is localised in the pronuclei of the early embryo (Bellier *et al.*, 1997). Furthermore, at the onset of the major-zygotic genome activation, the RPB1 becomes hyperphosphorylated by TFIIF kinase. These experiments suggested that the RPB1 could potentially regulate the onset of zygotic genome activation (Bellier *et al.*, 1997). Even though the main role of RNA Polymerase II is transcription, it is also implicated in other processes which will be discussed later.

Transcription alone is not sufficient to yield the appropriate mRNA coding sequence to express functional proteins. The process of splicing is an essential step after transcription and before translation that is necessary for the expression

of functional proteins. Up-regulation of transcripts for splicing and mRNA processing at the major and minor phases of zygotic genome activation suggest that this is an important developmentally regulated event.

1.3 The process of Splicing

Most genes in higher eukaryotes contain several intervening sequences, called introns that interrupt the coding sequence of the gene. In humans for example, an average gene is 27kb long, with an average of 8.8 exons per gene, each exon having a mean size of 145 nucleotides. This means that there is an average of 7.8 introns per human gene (Lander *et al.*, 2001). These introns need to be precisely excised from the pre-messenger RNA (pre-mRNA) to obtain mature mRNA with the appropriate coding sequence. After intron excision, the two flanking sequences are ligated together using sequential trans-esterification reactions. This splicing process is achieved by a multi-component complex that is called the spliceosome. The main components of the spliceosome are five uridine rich small nuclear ribonucleoprotein particles (UsnRNPs or snRNPs) as well as a number of other splicing factors mainly belonging to the SR protein family (Gall, 2000;Patel and Steitz, 2003;Shin and Manley, 2004). It has been believed for years that splicing is a process that takes place only in the nucleus of the cell. A recent study in anucleate platelets revealed that splicing can also take part in the cytoplasm (Denis *et al.*, 2005).

1.3.1 SnRNP biogenesis

The major spliceosomal snRNPs are U1, U2, U4, U5 and U6. The biogenesis of these molecules is a complex process and many aspects of it remain poorly understood. The snRNA of these precursors of these proteins with the exception of U6 are transcribed by RNA Polymerase II and contain additional 3' nucleotides. While the snRNA is in the nucleus it acquires a monomethylated m⁷GpppG cap structure, which is required to facilitate export from the nucleus (Hamm and Mattaj, 1990). In the cytoplasm, the UsnRNA then acquires a complex of seven Sm proteins, B/B', D3, D2, D1, E, F and G in a stepwise manner, to form the snRNP Sm core structure (Will and Luhrmann, 2001). The SMN complex also plays an essential role in the assembly of the snRNPs (Gubitz *et al.*, 2004) due to its capacity to bind to both Sm proteins and snRNAs. The SMN complex comprises of the survival of motor neurons protein (SMN) and at least six other proteins named Gemins (Gubitz *et al.*, 2004). The m⁷G cap of the snRNA is then converted to the 2,2,7-tri-methylated guanosine (m³G), by trimethyl guanoside synthase I (Tgs1p) (Colau *et al.*, 2004). The last process of the cytoplasmic maturation of the snRNA is that of removal of the extra ~20 nucleotides transcribed on the 3' end of the snRNA (Neuman, V and Dahlberg, 1990). The m³G cap is then recognised by Snurportin-1 (Huber *et al.*, 1998) and importin-β (Palacios *et al.*, 1997; Huber *et al.*, 1998), two factors that are required for the snRNP import. The final maturation processes that occur in the nucleus are the pseudouridylation and the 2'-O-methylation, followed by the association of

the UsnRNP specific proteins, U1-A and U2-B'. However, the factors involved in these processes are currently unknown (Will and Luhrmann, 2001).

The five snRNPs are synthesised both in the nucleus and the cytoplasm, while UsnRNP U6 is synthesised entirely in the nucleus by RNA Polymerase III and acquires a different cap structure known as γ -monomethyl cap structure. The other main difference with the other snRNPs is that in place of the Sm proteins, U6 associates with the Sm like proteins, LSm2, LSm3, LSm4, LSm5, LSm6, LSm7 and LSm8. The pseudouridylation and the 2'-O-methylation processes remain the same as those discussed earlier (Will and Luhrmann, 2001).

The final stage of maturation of snRNPs occurs in the cajal bodies, also known as coiled bodies. In these structures the snRNPs are also temporarily stored (Gall, 2000) (also see 1.3.4).

1.3.2 Spliceosome assembly and function (Major-class pathway)

As mentioned earlier, the splicing reaction is performed by the spliceosome. The key players of the spliceosome are the five snRNPs and a number of non-snRNP proteins, such as SR proteins (see section 1.4). The splicing process is achieved by a series of RNA-RNA, RNA-protein and protein-protein interactions (Hastings and Krainer, 2001b).

The diagram below shows the consensus sequences of the major and minor pathway introns and branch points. These are the typical sequences that are recognised by the splicing machinery in the 5'splice site, the 3' splice and the

branch point. In the diagram below (Figure 1.1), the size of its letter demonstrates the percentage occurrence of the nucleotide in the particular position, i.e. a full size letter represents 100% occurrence in the particular position. The vertical lines in the diagram also show the sites where the mRNA is excised.

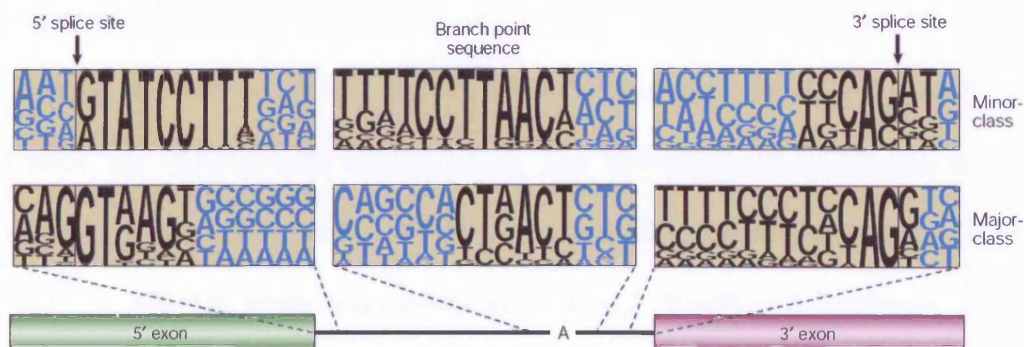
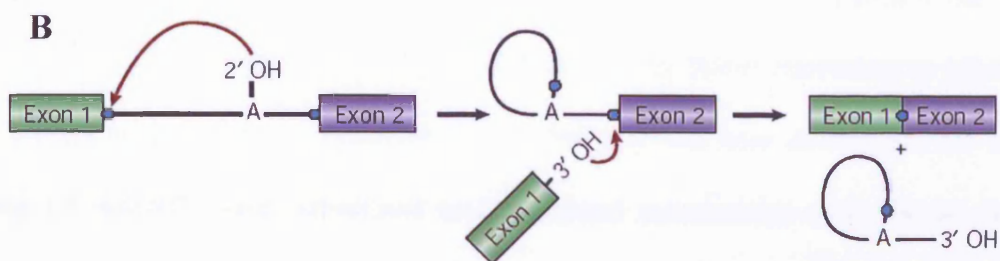
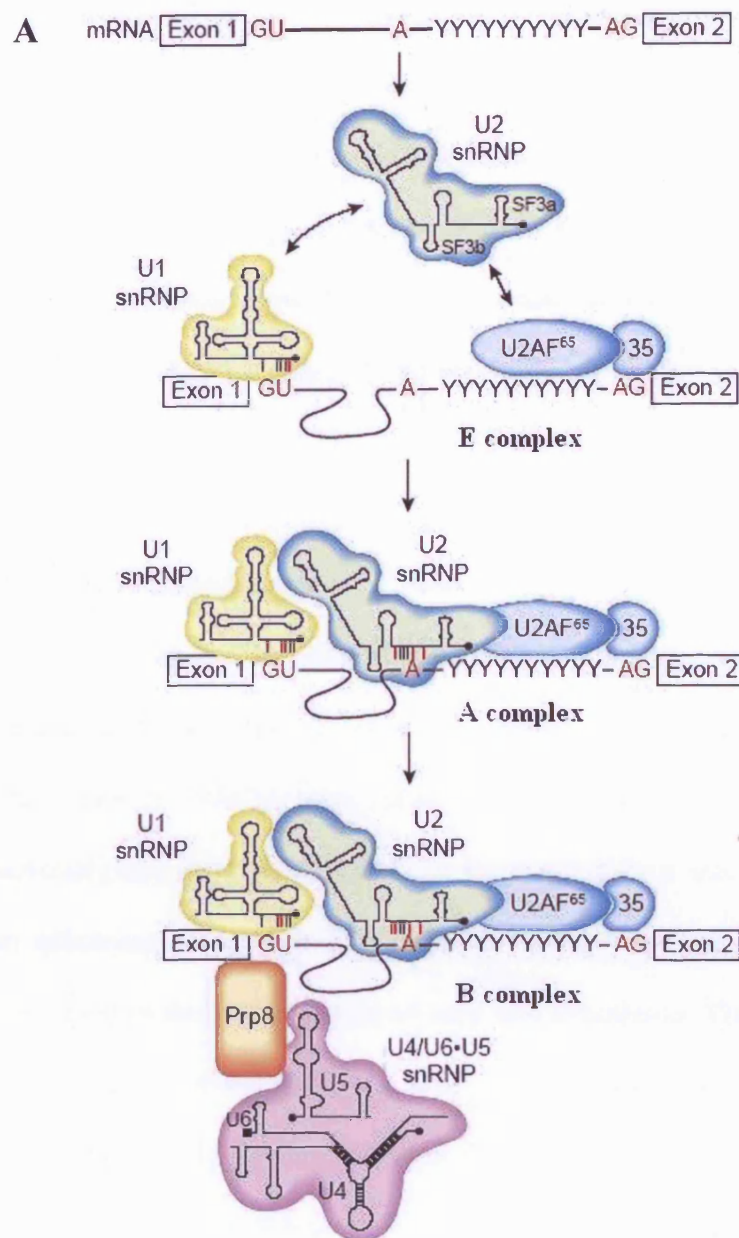


Figure 1.1. The consensus sequences of the 5' splice site, 3' splice site and branch point involved in both the major as well as the minor class splicing pathway. The size of each letter demonstrates the percentage occurrence of the nucleotide in the particular position. The 5' and 3' splice sites show the excision points on the mRNA. Finally the relative position of these three consensus sequences within the intron are also shown. From (Patel and Steitz, 2003).

The mature spliceosome is built in a stepwise manner that has been broken down to series of complexes. There are four distinct complexes on the way to form the mature spliceosome. These complexes are E, A, B (shown in figure 1.2), and C in that order. The E complex is formed by the attachment of the U1 snRNP to the 5' splice site (Mount *et al.*, 1983; Seraphin and Rosbash, 1989). A recent study demonstrates that SF1 that binds to the branch site following the U1 snRNP 5' splice site recognition (Kent *et al.*, 2005), compared to earlier studies implicating the function of SF1 at the A complex (Berglund *et al.*, 1997). Furthermore, the

attachment of the U2 auxiliary factor (U2AF) to the pre-mRNA occurs at this stage. The U2AF is a heterodimer of U2AF₆₅ and U2AF₃₅ that bind to the polypyrimidine tract found before the 3' splice site and 3' splice site, respectively (Ruskin *et al.*, 1988). At the same time another essential splicing factor, UAP56, that plays a role later in mRNA export is recruited to the spliceosome in a manner dependent on U2AF₆₅ (Fleckner *et al.*, 1997). In the A complex the U2 snRNP (Zhuang and Weiner, 1989) as well as Splicing Factor 3b (SF3b) bind to the branch site (Kramer and Utans, 1991). The tri-snRNP of U4/U6•U5 is then recruited to the spliceosome to form the complex B (Konarska and Sharp, 1987), with U4 and U6 being extensively base paired (Wolff and Bindereif, 1993). Structural changes in U4 and U6 result in juxtaposition of the 5' and 3' splice sites, and the release of the U1 and U4 (Lamond *et al.*, 1988). U6 then forms a base pair bond with the 5' splice site (Wassarman and Steitz, 1992) and the 3' of U6 base pairs to the 5' of U2 (Wu and Manley, 1991). The cleavage of the 5' exon-intron junction occurs due to the nucleophilic attack from a conserved adenosine residue at the intron's branch site. This step generates a free 3' hydroxyl group and the 5' exon as well as a branched lariat intermediate (Moore and Sharp, 1993). In the last complex, C, U5 base pairs to both the 5' and 3' splice sites bringing them together. The second step of splicing occurs by the 3'



exon being attacked by the 3' hydroxyl group found in the 5' exon. At the same time the lariat intron is released completing the splicing process (Newman, 1997). However, an alternative model of the U5 function has also been proposed. U5 was proposed to take part in 5' splice site selection in collaboration with U1 and the conserved splicing factor Psp8 before the binding of U2 to the branch point (Wyatt *et al.*, 1992; Maroney *et al.*, 2000). It is currently not yet clear which of the two models is correct.

1.3.3 Minor-splicing pathway

The major class pathway of splicing described above recognises introns with highly conserved dinucleotides in the intron termini, GT for the 5' end and the AG for the 3' end. In 1994 however, introns with non-consensus splice sites were first discovered (Hall and Padgett, 1994). In the same study it was also shown that a distinct spliceosome was responsible for the splicing of such introns and the snRNPs involved in this process were of very low abundance. The 5' splice sites and 3' splice sites that involved this new pathway had termini AT and AC respectively. The two newly identified snRNPs were shown to be U11 and U12 with properties mimicking those of U1 and U2 described earlier (Montzka and Steitz, 1988). Furthermore, there was no polypyrimidine tract upstream the 3' splice site in these introns and therefore there was no factor equivalent to U2AF involved in their splicing (Dietrich *et al.*, 1997). It was later shown that the U4 and U6 snRNPs were substituted in this second spliceosome with U4atac and

U6atac (the *atac* denotes the different termini) (Tarn and Steitz, 1996). Finally, the U5 snRNP serves as a factor involved in both spliceosomes. This suggests that U5 does not base pair with sequences that are different in genes spliced by the two different spliceosomes. The role of U5 in the minor-class pathway is to form the U4atac/U6atac_oU5 tri-snRNP (Schneider *et al.*, 2002) (an analogue of U4/U6_oU5) and to recruit other protein factors which are important in this splicing pathway (Patel and Steitz, 2003). The occurrence of introns that are spliced by the minor-class pathway of splicing varies in animal between 1 in 100 to 1 in 300 genes (Patel and Steitz, 2003).

1.3.4 Spatial Organisation of UsnRNPs and other splicing factors

Early immunofluorescence experiments localised UsnRNPs to the nucleus of the cell but the studies did not yield any information as to how they were organised in the nucleus and in which structures transcription or splicing take place (Lobo *et al.*, 1988). Later experiments where snRNPs were tagged with fluorescent proteins, yielded more information to how these structures are organised in the nucleus. Newly synthesised snRNPs localise firstly to the coiled or Cajal bodies (Sleeman and Lamond, 1999). Cajal bodies are nuclear spherical structures of 0.3-1µm diameter that act as storage or maturation sites for transcription and splicing factors. However, transcription and splicing do not take place in the Cajal bodies (Gall, 2000). SnRNPs localise in nuclear “speckles”. These are spherical structures between 0.8-1.8µm in diameter consisting of snRNPs, SR proteins (see

section 1.4), other splicing and transcription factors and 3' end maturation proteins (Mintz and Spector, 2000). Furthermore, it has been reported that phosphatidylinositol phosphate kinases and phosphatidyl inositol bisphosphate (PIP₂) localise in these structures, suggesting that these lipids and their lipid kinases may be involved in regulation of splicing (Boronenkov *et al.*, 1998). These speckle structures are otherwise known as interchromatin granule clusters (IGCs). IGCs appear to be important in the assembly and modification of splicing factors, but the fact that RNA Polymerase II does not co-localise in the same structure suggests that transcription and splicing do not take place in these structures (Mintz and Spector, 2000). Perichromatin fibrils (PFs) are structures found in close proximity to the IGCs and also scattered within the nucleus (Spector, 2001). These are the areas where transcription and its related processes take place (see section 1.3.5) (Parfenov *et al.*, 2003). The factors required for these processes are recruited from the neighbouring IGCs. Upon completion of transcription, these factors return to the IGCs. IGCs are important structures for transcription and pre-mRNA splicing, and their disassembly dramatically reduces mRNA splicing and inhibits the coordination of transcription with splicing (Sacco-Bubulya and Spector, 2002). Finally, it has been shown that splicing factors such as SC35 and RNA Polymerase II localise in the cajal bodies in transcriptionally inert cell types, such as antral follicles (Parfenov *et al.*, 2003).

It has been believed for years that splicing takes part only in the nucleus of the cell, as described above. A recent study in anucleate platelets has revealed that

splicing can also take place in the cytoplasm (Denis *et al.*, 2005). Even though these platelets do not possess a nucleus, splicing factors have been shown to exist and function in the cytoplasm of the cell.

1.3.5 Integration of transcription, capping, polyadenylation, and nuclear export with splicing.

Most biology textbooks still present splicing to occur after transcription is completed. However, splicing has been shown to occur cotranscriptionally and is tightly coupled to RNA polymerase II (Bentley, 2005). It has been recently shown that splicing and polyadenylation can be completed by the time that the polymerase has transcribed 1kb beyond the processing sites and such a process can occur in 30 seconds in vivo. However, in vitro where splicing is uncoupled from transcription the process takes place more slowly and may take hours (Wetterberg *et al.*, 2001).

The regulation of transcription and the associated processes mentioned above are controlled by the C-terminal domain (CTD) of the large subunit of RNA polymerase II, also known as RPB1 or LS. The importance of the CTD is shown in experiments where truncation of the CTD causes defects in capping, polyadenylation and splicing (McCracken *et al.*, 1997; Misteli and Spector, 1999). The important sequence in the CTD is a series of Y₁S₂P₃T₄S₅P₆S₇ heptad repeats. There are 27 tandem heptad repeats in the yeast and 52 in humans. However, the

in vivo functional unit requires only a pair of these tandem repeats (Stiller and Cook, 2004).

Phosphorylation of particular amino acids of the heptad repeats regulates the integration of other processes with transcription. After transcription initiation, the serine 5 of the CTD heptad repeat is phosphorylated by the TFIIF-associated kinase (Cho *et al.*, 2001). This phosphorylation removes the initiation factors from the pre-mRNA complex and recruits the capping machinery. Capping protects the mRNA from degradation and occurs when the pre-mRNA is only 20-40 nucleotides long. Serine 5 is subsequently dephosphorylated to release the capping machinery (Komarnitsky *et al.*, 2000).

The elongation factors are then recruited by P-TEFb, in a manner dependant on phosphorylation of Serine 2 of the heptad repeat (Maniatis and Reed, 2002). One of these recruited factors is TAT-SF1, which is responsible for recruiting the spliceosomal U small nuclear ribonucleoproteins (snRNPs) to the nascent mRNA and strongly stimulates transcription elongation as well as splicing (Fong and Zhou, 2001). Further evidence to support the connection between transcription elongation and splicing is that genes containing introns are transcribed more efficiently (McCracken *et al.*, 1997; Fong and Zhou, 2001).

The 3' end maturation of the newly transcribed mRNA is also tightly controlled by the CTD phosphorylation status. Serine 2 phosphorylation of the CTD leads to mRNA maturation of the 3' end including the cleavage of the

nascent mRNA 20-30 bases downstream of a conserved poly A site and the polyadenylation by polyA polymerase (PAP) (Proudfoot, 2004).

Finally, transcription is indicated to be involved with splicing and nuclear export of the newly transcribed and processed mRNA. Even though nuclear export occurs after 3' mRNA maturation, it has been indicated that it is coupled to splicing. The Transcription/Export (TREX) complex of proteins is constituted by the THO complex of proteins, which consists of five proteins (their properties are yet to be identified), UAP56 and Aly (Reed and Cheng, 2005). This complex of proteins is involved in the nuclear export of the mRNA. The splicing factor UAP56 described earlier, recruits the mRNA export factor Aly to the splicing complex (Zhou *et al.*, 2000; Luo *et al.*, 2001). This is demonstrated by experiments where UAP56 and Aly co-localise in the spliceosome (Custodio *et al.*, 2004). Following the splicing reaction, it is suggested that Aly binds to TAP, a protein that is involved in mRNA export by bringing the mRNA to the nuclear pore (Strasser and Hurt, 2001). However, the role of the THO part of the TREX complex is not yet clear. The fact that components of the THO associate with the spliced but not unspliced mRNA indicates its involvement in splicing and furthermore its coupling to transcription (Reed and Cheng, 2005).

1.4 SR proteins and splicing

The SR family proteins are essential splicing factors with multiple splicing roles in both major and minor splicing pathways (Hastings and Krainer, 2001a). Their main roles are splice site selection , promotion of the spliceosome assembly (Hertel and Graveley, 2005) and regulation of alternative splicing (Wang and Manley, 1995;Mayeda *et al.*, 1999).

1.4.1 SR protein properties and their involvement in splicing

SR proteins are conserved throughout the metazoa. Their N-terminal domain contains either one or two RNA binding domains that interact with the pre-mRNA whereas the C-terminal domain is the serine / arginine rich domain that acts as a protein interaction domain (RS domain) (Graveley, 2000).

The RNA-binding domain of the SR proteins can bind to the pre-mRNA in the absence of the RS domain (Caceres and Krainer, 1993;Tacke and Manley, 1995). However, deletion of the RS domain prevents binding of the SR protein to RNA Polymerase II RPB1, possibly disturbing the integration of transcription and splicing (Misteli and Spector, 1999). The RNA-binding domain has been shown to bind to exon sequences and enhance the splicing of the adjacent exon. These exon sequences are called exonic splicing enhancers (ESEs) and can be found either upstream of the 5' splice site or downstream of the 3' splice site. Following the binding of the RNA binding domain in the ESE other splicing factors are recruited to the site of splicing.

In the case of the ESE found downstream of the 3' splice site, the RS domain of the SR protein interacts with U2AF₃₅ and recruits it to the 3' splice site. U2AF₆₅ and the rest of the spliceosome are then recruited to the splice site. There is evidence that such a recruitment of the spliceosome can be facilitated by SC35 or SF2/ASF in a RS-domain dependant manner (Wu and Maniatis, 1993).

It was shown in earlier studies that when an ESE is found upstream the of 5' splice site, the bound SR protein ASF/SF2, interacts with the 70 kDa of the U1 snRNP and stimulates the binding of the U1 snRNP to the downstream 5' splice site (Wu and Maniatis, 1993). It was later shown that such an interaction does not actually occur, but ASF/SF2 cooperates with U1 and enhances the recognition of the 5' splice site (Kohtz *et al.*, 1994;Cao and Garcia-Blanco, 1998). A further role of the SR proteins is to recruit the U4/U6•U5 tri-snRNP into the spliceosome (Roscigno and Garcia-Blanco, 1995). However, the precise role in this activity is not yet known. Two novel SR proteins, U4/U6•U5-27K (Fetzer *et al.*, 1997) and U5-100K (Teigelkamp *et al.*, 1997) were later identified on the tri-snRNP as candidates for this activity, but an RNA binding domain for these proteins has not yet been identified.

Recently, a completely different model of the RS domain function has been presented. It is proposed that the ESE-bound SR protein does not bind with its RS domain to an UsnRNP or to U2AF as earlier described but to the branch site, in a process that promotes the pre-spliceosome assembly (Shen *et al.*, 2004). It was later shown by the same group that prior to pre-spliceosome assembly, the

RS domain of the U2AF₆₅ binds to the branch site by its RS domain during the formation of complex E. Finally, a third SR protein binds by its own RS domain to the 5' splice site to complete assembly of the mature spliceosome (Shen and Green, 2004). The group suggests that this model fits with earlier data of 5' splice site selection by ASF/SF2 (Zuo and Manley, 1994) or the promotion of the U1 5'splice site interaction (Eperon *et al.*, 1993), but such a model has not yet been confirmed by other groups.

It was recently shown that SR proteins are also involved in the minor-class splicing pathway. In this study by Hastings and Krainer, it was shown that the SR proteins are involved in the formation of splicing complex A and therefore are involved in the assembly of the second spliceosome. Addition of recombinant 9G8 or SRp55 activated splicing of a minor-class pathway intron in the SCN4A gene. However, the two main SR proteins SC35 and ASF/SF2 could not activate splicing of this gene. However, when an exonic splicing enhancer was introduced on the gene, ASF/SF2 but not SC35 was able to activate splicing, presenting a novel role of the SR proteins (Hastings and Krainer, 2001a).

1.4.2 Novel Roles of the SR proteins

SR proteins were considered for years to be specifically involved in splicing, but recently three novel roles of the SR proteins were demonstrated.

Firstly, they were shown to associate with intronless or spliced mRNAs and facilitate their export to the cytoplasm (Huang and Steitz, 2001; Masuyama *et*

al., 2004). However, only proteins such as SRp20, 9G8 and ASF/SF2 that are shuttling between the nucleus and the cytoplasm (Caceres *et al.*, 1998) can take part in this pathway. On the other hand, SC35 that does not shuttle between the nucleus and the cytoplasm due to a strong nuclear retention signal on its RS domain and as such, does not take part in mRNA nuclear export (Cazalla *et al.*, 2002). The export of the mRNA is facilitated by the recruitment of the general export receptor TAP, to the mRNA by the SR protein, which later associates with the Aly family or export adapter proteins that export the mRNA (Huang and Steitz, 2005).

Secondly, shuttling SR proteins and in particular SF2/ASF was shown to be involved in mRNA translation. SF2/ASF associates with translating ribosomes and can stimulate translation even if splicing was not earlier required. The shuttling ability of the protein was also shown to increase when an exonic splicing enhancer was present (Sanford *et al.*, 2004). Even though the precise role of the SR protein in translation is not yet identified, mRNA synthesis, processing and now translation appear to be linked.

Thirdly, SR proteins have been shown to be involved in mRNA surveillance and quality control. Overexpression of SR proteins such as SF2/ASF or SC35 had the effect of increasing the targeting of mRNAs with premature termination codons to non-sense mediated decay (NMD) (Zhang and Krainer, 2004), a process of degradation of mRNAs containing stop codons in regions

close to ~25 nucleotides upstream of a splicing generated exon (Lejeune and Maquat, 2005).

1.4.3 Regulation of SR proteins

SR protein localisation and function are regulated by phosphorylation both for their splicing and nuclear shuttling activity. SR proteins are active when phosphorylated in their serine residues and are recruited from the nuclear speckles to the spliceosome for the splicing reaction to take place (Misteli *et al.*, 1998; Xiao and Manley, 1998). During splicing, they become partly dephosphorylated and return to the speckles where another round of phosphorylation and activation takes place (Misteli *et al.*, 1998). Dephosphorylation of SF2/ASF was shown not only to repress splicing, but its dephosphorylation was shown to be essential for the first trans-esterification reaction of splicing (Cao *et al.*, 1997). In contrast, dephosphorylation of other SR proteins such as SRp38 was shown to inhibit splicing in HeLa cells (Shin and Manley, 2002). At the same time, dephosphorylation is important in shuttling proteins such as ASF/SF2, 9G8 or SRp20, in order to shuttle from the nucleus to the cytoplasm (Caceres *et al.*, 1998; Huang *et al.*, 2004).

However, SR proteins with a strong nuclear retention signal, such as SC35 or SRp40, do not shuttle between the nucleus and the cytoplasm (Caceres *et al.*, 1998). The difference between shuttling and non-shuttling proteins lies in the RS domain of these proteins (Caceres *et al.*, 1998; Cazalla *et al.*, 2002). The strong

nuclear retention signal on SC35 was shown to be in a 30 amino acid proline rich segment at the C-terminus of its RS domain. In the same study it was shown that 10 consecutive RS dipeptides on SF2/ASF are enough for normal localisation and splicing activity of the SR proteins, compared to the wild type form of the protein, where the same number of dipeptides is scattered through 31 more amino acids (Cazalla *et al.*, 2002). After shuttling to the cytoplasm in the dephosphorylated form, phosphorylation is required for nuclear import (Lai *et al.*, 2000).

SR protein phosphorylation and localisation are also important in early development. In the early nematode embryo, SR proteins are found in the cytoplasm in their hyperphosphorylated form. Following zygotic genome activation, the proteins become partially dephosphorylated, to reach an intermediate stage of phosphorylation and localise in the nucleus of the cell (Sanford and Bruzik, 1999; Sanford and Bruzik, 2001).

There are three different protein families that can phosphorylate the RS domains of SR proteins. These are the SR protein kinase family (SRPK 1 and SRPK2), Clk/Sty family and the DNA topoisomerase I. SF2/ASF was shown to be phosphorylated by SRPK1 and SRPK2. The two kinases were both localised in the nucleus and the cytoplasm, suggesting the role of phosphorylation both in activation of splicing within the nucleus and the role of cytoplasmic phosphorylation for return of the shuttling SR proteins back to the nucleus (Wang *et al.*, 1998; Koizumi *et al.*, 1999; Aubol *et al.*, 2003). The Clk/Sty kinase was also shown to phosphorylate SR proteins, but it only localises in the nucleus of the

cell. Furthermore, hypo-phosphorylation or hyper-phosphorylation of the SR proteins by the Clk/Sty can cause inhibition of splicing and also alternative splice site selection (Prasad *et al.*, 1999; Prasad and Manley, 2003). Finally, DNA topoisomerase I can also phosphorylate SR proteins and potentially regulate pre-mRNA splicing. DNA topoisomerase depletion experiments demonstrated hypo-phosphorylation of the SR proteins and alternative splice sites being selected due to impairment of the exonic splicing enhancer splicing (Rossi *et al.*, 1996; Soret *et al.*, 2003). The only phosphatase believed to be involved in at least one dephosphorylation event during spliceosome assembly is PP2c γ . The precise mechanism of its role still remains to be investigated (Murray *et al.*, 1999).

1.5 Alternative splicing

Alternative splicing is an important mechanism that contributes significantly to the regulation of gene expression. Alternative splicing can regulate protein function by inclusion or exclusion of a particular domain in the mRNA. Furthermore, it can regulate gene expression by producing spliced transcripts that are susceptible to mRNA degradation (Lareau *et al.*, 2004). A recent study that analysed 10,000 human genes shows that at least 74% of the investigated genes were alternatively spliced (Johnson *et al.*, 2003).

1.5.1 Types of alternative splicing

There are five different types of alternative splicing. The most common one with a 38% of occurrence in the alternative splicing events is the exon skipping. The second most common alternative splice events are those of alternative 5' or 3' splice site selection with occurrence 18% and 8% respectively over the total amount of alternative splice site events. Intron retention accounts for 3% of alternative splicing events. Finally, the remaining occurrence includes more complex events that consist of mutually exclusive events, multiple polyadenylation sites and alternative transcription sites (Ast, 2004).

1.5.2 Regulation of Alternative Splicing

Alternative splicing can be regulated by four distinct elements that are found in the mRNA. These include Exonic Splicing Enhancers (ESEs), Exonic Splicing Silencers (ESSs), Intronic Splicing Enhancers (ISEs) and Intronic Splicing Silencers (ISSs) (Garcia-Blanco *et al.*, 2004).

ESEs are sequences that are recognised by SR proteins. Upon binding of the SR protein to the ESE, the splicing machinery is recruited for splicing to take place. Examples of SR proteins that possess such properties are SF2/ASF (Bourgeois *et al.*, 1999) or SC35 (Liu *et al.*, 2000). Finally, more than 50% of the mutations involved in exon skipping are due to mutations found in ESEs that affect the target motifs of SR proteins (Liu *et al.*, 2001).

ESSs however repress the splicing of a particular exon. This repression is facilitated by specific proteins of the hnRNP family. These proteins bind to the ESSs and compete with U2AF₆₅ for binding to the polypyrimidine tract, and therefore inhibit the assembly of the spliceosome (Domsic *et al.*, 2003). An example of such a protein is hnRNP A1 which has been also shown to antagonise other SR proteins for exon definition (Caceres *et al.*, 1994; Eperon *et al.*, 2000). In overexpression studies, SF2/ASF overexpression favours the proximal 5' splice site, whereas overexpression of hnRNP A1 favours the distal 5' splice site (Zhu *et al.*, 2001).

Intronic enhancer sequences have been shown to enhance splicing of the 5' splice site found in the vicinity of the enhancer sequence. The first one is UGCAUG, which is found predominantly in the brain or neural tissues (Minovitsky *et al.*, 2005). The selection of this intron enhancer sequence is mediated by Fox-1, but the mechanism by which splicing is mediated is not yet known (Nakahata and Kawamoto, 2005). Another intronic enhancer sequence is that of GGG which is suggested to recruit U1 snRNP to the 5' splice site (McCullough and Berget, 2000). A protein that also has been involved in the recognition of intronic splicing enhancers is hnRNP K, but its involvement with the spliceosome assembly is yet to be identified (Expert-Bezancon *et al.*, 2002). Nova-1 is finally another example of a protein involved in regulation of splicing by recognising UCAU repeats mainly in neuronal tissues (Buckanovich and Darnell, 1997).

Finally, intronic splicing silencers inhibit splicing of the particular intron and have an effect on alternative splicing. The size of these sequences varies but they are found in close proximity with the polypyrimidine tract. These sequences mainly have an effect in spliceosome assembly (Wagner *et al.*, 2005). An example of intronic splicing inhibition is that of Polypyrimidine tract binding protein (PTB), which upon binding to the polypyrimidine tract binding site CUGUGC (Spellman *et al.*, 2005), inhibits splicing by preventing the assembly of the U2AF on the 3' splice site (Sharma *et al.*, 2005).

1.5.3 Regulation of gene expression by alternative splicing

Down-regulation of a gene can be achieved in the cell by a mechanism of mRNA surveillance called nonsense-mediate decay (NMD). NMD involves the recognition of a premature termination codon (PTC) which is localised ~25 nucleotides upstream of splicing generated exon junction (Lejeune and Maquat, 2005). Many genes have been identified that are regulated by the incorporation of a PTC in the mRNA. Alternative splicing that can lead to PTC can be either due to mis-spliced products or for down-regulation of a gene. An example of a gene with mis-spliced transcript products that have an incorporated PTC that is degraded by NDM is the Fibroblast Growth Factor Receptor 2 (FGFR2) gene (Yeh *et al.*, 2003). On the other hand auto-down-regulation of the gene by NMD has been shown to occur in the SR-like protein Tra2-beta (Stoilov *et al.*, 2004).

1.5.4 Alternative splicing and Drosophila Sex determination

The best example that demonstrates the importance of alternative splicing in the regulation of gene expression is in the sex determination pathway in *Drosophila*. The sex determination pathway in *Drosophila* is based on a series of alternative splice site events.

The first gene that is alternatively spliced is the Sex-Lethal (Sxl) gene. The Sxl gene is expressed shortly after fertilisation by an early promoter found only in the female. The gene is alternatively spliced excluding exons 2 and 3. This expression however does not occur in the male. The maintenance promoter found in both males and females will drive the expression of the Sxl protein in both males and females. The presence of the Sxl protein early in development leads to alternative splicing of the Sxl gene produced by the maintenance promoter in females that yields a functional protein, by splicing out exon 3. Exon 3 is an unusual exon of the Sxl gene because it contains a stop codon. On the other hand in the male, a truncated non-functional protein is expressed, due to the absence of the Sxl expressed early in development. The presence of the Sxl protein in females then regulates the alternative splicing of tra2, a splicing regulation factor, by splicing out yet another stop codon from exon 2 and leading to the production of a functional protein. Due to the absence of the Sxl in the males a truncate non-functional form of tra2 is expressed. The presence or absence of tra2 then regulates the alternative splicing of the doublesex (dsx) gene that determines the sex of the offspring. In the females, the protein contains exons 1 through 4. In the

males however, the protein contains exons 1 through 6 but exon 4 is spliced out (Pomiankowski *et al.*, 2004).

1.5.5 SR proteins and alternative splicing

Another property of the SR proteins is to affect alternative 5' and 3' splice site selection. Overexpression of both SF2/ASF and SC35 favours proximal over distal 5' splice site selection. Furthermore, this property is antagonised in both cases by heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (Krainer *et al.*, 1990;Caceres *et al.*, 1994). Both SF2/ASF (Eperon *et al.*, 1993;Sun *et al.*, 1993) and SC35 (Liu *et al.*, 2000;Zahler *et al.*, 2004) have been shown to facilitate such a selection by binding to exonic splicing enhancer and therefore recruit the UsnRNPs to the site of splicing. In the case of SF2/ASF, the recognition property of the proximal 5' splice site lies in the RNA Recognition Motif (RRM) 2 and not in the RS domain (van Der Houven Van Oordt *et al.*, 2000). Proximal over distal splice site selection also occurs in the 3' end of the splice site, an effect however that is not antagonised by the hnRNP A1 (Fu *et al.*, 1992). Finally, SC35 can autoregulate its own expression by alternative splicing. Overexpression of SC35 led to an exon exclusion and an intron excision in the 3' untranslated region on its endogenous mRNA leading to the production of a very unstable mRNA molecule that was degraded (Sureau *et al.*, 2001), leading to a lower production of endogenous SC35.

1.5.6 Alternative splicing and disease

Alternative splicing of a gene due to a mutation on an intron site or branch site or due to abnormal expression of a splicing factor can lead to disease. Approximately 15% of mutations that cause genetic disease affect splicing (Krawczak *et al.*, 1992). Examples of disease caused by such splicing abnormalities include β -thalassemia, myotonic dystrophy, spinal muscular atrophy, retinitis pigmentosa, atypical cystic fibrosis, Frasier syndrome and even malignancy (Faustino and Cooper, 2003; Garcia-Blanco *et al.*, 2004). Furthermore regulation of splicing is essential for normal heart development (Cooper, 2005; Ladd *et al.*, 2005), brain development (Lins and Borojevic, 2001), kidney development (Charlet-Berguerand *et al.*, 2004), and skeletal muscle development (Cheema *et al.*, 2005).

SR proteins are often the cause of disease or the cause of abnormal development in some cases. Some examples of the SR protein involvement in alternative splicing are given below. ASF/SF2 was shown to be an essential factor for normal heart development. Cardiomyocytes deficient in ASF/SF2, produce an alternatively spliced calmodulin-dependant kinase $\text{Ii}\delta$, which lead to hypercontraction of the heart due to abnormal development (Xu *et al.*, 2005).

SC35 has also been involved in disease and development. SC35 deletion from the thymus, leads to alternative splicing of CD45 which causes a defect in the T-cell maturation due to the thymus only developing to 10-30% of its normal size (Wang *et al.*, 2001). SC35 is also suggested to be involved in certain cases of Alzheimer's

disease. It is suggested by Hernandez and colleagues that SC35 is involved in the aberrant splicing of tau exon 10, of the glycogen synthase kinase 3 (Hernandez *et al.*, 2004).

Finally, SR proteins and alternative splicing are implicated in breast and ovarian cancers. Alternative splicing of CD44 is believed to be caused by Tra2beta, SC35 and ASF/SF2, which are shown to be overexpressed in these cases of cancer (Fischer *et al.*, 2004).

1.6 Synopsis

In this introduction I have highlighted the mechanisms of mRNA splicing. The experiments that follow present the development and characterisation of a fluorescent probe that allows the measurement of splicing efficiency in single cell embryos. These two particular SR proteins were selected to be investigated due to their opposing functions. SC35 is an activator of splicing and is involved in enhancer-dependent splicing. The organisation of SC35 in nuclear “speckles” as well as their involvement in splicing were investigated. Following the construction of the M1M2 probes, the monitoring of enhancer-dependent splicing could be investigated, but failure to deplete SC35 from embryos did not allow such experiments to be performed. On the other hand SRp38 is a potent inhibitor of splicing. Its role in early development was investigated by the overexpression of a fusion SRp38-EGFP protein or by SRp38 depletion using morpholinos. However, the expected splicing inhibition was not observed during SRp38 overexpression.

2. Materials and methods

2.1 Oocyte and embryo collection

Immature (germinal vesicle stage) oocytes were retrieved from the ovaries of 21-24 day old female MF1 mice that had been administered a 7 IU intraperitoneal injection of pregnant mare serum gonadotrophin (PMSG; Intervet) 48 hours earlier. Mice were kept in a light/dark cycle with free access to food and water. Prior to removal of the ovaries, the mice were culled by cervical dislocation. Ovaries were placed in warmed HEPES-buffered KSOM medium (H-KSOM) (Summers *et al.*, 2000) containing 1mg/ml fraction V BSA (Sigma Chemicals, Poole, Dorset, UK) with 200 μ M IBMX (Sigma UK) to prevent germinal vesicle breakdown, and maintained at 37°C. Oocytes were recovered by puncturing the surface of the ovary with a 27-gauge needle, collected using a mouth-operated pipette, and placed in drops of media under oil to prevent evaporation (Mineral oil; embryo tested, Sigma UK). Only oocytes with an intact layer of cumulus cells were recovered, and cumulus cells were subsequently removed by repeated pipetting with a narrow pipette.

To recover mature (MII) oocytes, human Chorionic Gonadotrophin (hCG; Intervet) was administered 48-54 hours after PMSG. Mice were culled and oviducts removed 14-16 hours post-hCG. Cumulus masses were released into H-

KSOM by tearing the oviduct using forceps. Cumulus cells were removed by addition of hyaluronidase (300µg/ml; embryo tested grade, Sigma UK) to the media. Following recovery, oocytes were washed through at least three drops of hyaluronidase-free HKSOM under oil.

For recovery of pronucleate embryos, female mice were mated with males at the time of hCG administration. The embryos were recovered from the oviduct by the same method as for mature eggs, 26-28h after hCG and mating. Embryos at the two-cell stage were recovered 48 hours after hCG and mating.

Oocytes and embryos that were not immediately utilised were left to undergo oocyte maturation and embryo development, respectively, in KSOM at 37°C in an atmosphere of 5% CO₂ in air.

2.2 Microinjection

Embryos were microinjected at 26 hours post hCG (pronucleus stage) or at ~50 hours post hCG (middle 2-cell stage). They were placed in a drop of HEPES-buffered KSOM under oil and pressure injected with a micropipette and Narishige manipulators mounted on an inverted microscope (Leica, Wetzlar, Germany). A holding pipette was used to immobilise the embryos and the injection pipette was pushed until inside the nucleus of one of the blastomeres. A brief overcompensation of negative capacitance was used to penetrate the plasma and nuclear membranes. A fixed pressure pulse was used for microinjection, using a picopump (WPI, Sarasota, FL). The same technique was performed for the

cytoplasmic injections. However, the injection pipette was only pushed through the zona pellucida and in contact with the plasma membrane before negative capacitance was used. The picopump was set up to deliver an injection of ~ 5% of cell volume of the vector in the nucleus and ~10% of the cell volume of mRNA or oligos in the cytoplasm.

After microinjection, the oocytes were removed to the hot block in fresh drops of H-KSOM under oil and allowed to recover for a few minutes before any other manipulation.

2.3 Probe construction

EGFP was amplified from base 616 to base 1330 using the pEGFP-C1 vector (Clontech, Inc., UK) as a template, the 5' primer GAC GCG GAT CCG TGA GCA AGG GCG AGG A and the 3' primer CAG GAT CTA GAT TAC TTG TAC AGC TCG TCC. The PCR product was subcloned into the pDsRed Express – C1 (Clontech, Inc., UK) in the restriction sites BamHI and XbaI. These restriction sites were incorporated in the primers. The TN24 intron was described by (Nasim *et al.*, 2002). The intron was amplified using the published vector as template and primers for 5' GAT CTC GAG TAC TCC CTC TCA AAA GCG GGC and for 3' GAC GCG GAT CCC TTC TCC GCC TGA GCC TCA. The amplified intron was subcloned into the DsRed Express – EGFP vector using the XhoI and BamHI restriction sites. The M1M2 and M1M2ΔE vectors were also subcloned in the DsRed Express – EGFP using restriction sites HindIII and

BamHI. The M1M2 intron was amplified using the 5' primer TAC CCA AGC TTT ACA AGG TAG TAT GTT GTG GGG CTG and the 3' GAC GCG GAT CCA ATA AGT TTA TAG ACT GCG GCC GA . The M1M2 Δ E was amplified by 5' primer TAC CCA AGC TTT ACA AGG TAG TAT GTT GTG GGG CTG and 3' primer GAC GCG GAT CCA ATA AGT TTA TAG ATG CTG AGA GT. The TN24 mutated intron was amplified without the 5' splice site using the 5' primer TAC CCA AGC TTT ACT CCC TCT CAA AAG CGG and the 3' primer CAG GAT CTA GAT TAC TTG TAC AGC TCG TCC. The intron was then subcloned using the HindIII and BamHI restriction sites. To ensure cloning success, all vectors were verified by sequencing performed by PNACL at Leicester University. All primers described in this thesis were prepared by Invitrogen UK or USA. (For sequences of these vectors see Appendix I).

2.4 Production of Probe mRNA

A PCR reaction was set up using the DsRedEx-TN24-EGFP vector as template, and primers 5' AAA TTA ATA CGA CTC ACT ATA GGG TCA GAT CCG CTA GCG CTA CCG and 3' CAG GAT CTA GAT TAC TTG TAC AGC TCG TCC. The 5' primer was designed to include the T7 polymerase promoter sequence upstream the start codon of the DsRed Express sequence. The 3' primer was designed to bind to the 3' end of the EGFP. The PCR product was then used as a template for an mRNA reaction using the mMessage mMachine T7 Ultra mRNA kit (Ambion, Texas, USA). The mRNA was polyadenylated as instructed

by the manufacturer and was then purified by Rneasy column (Qiagen). The mRNA was finally eluted in Rnase free water, at a concentration of 200-300ng/μl, which was the concentration used for microinjection.

2.5 Western Blotting

For Western blotting, 150 embryos were washed in PBS/BSA solution and then they were transferred in 4μl of SDS-sample buffer (0.125M Tris-HCl, 4% SDS, 20% glycerol, 10% mercaptoethanol, 0.002% bromophenol blue) making the total sample volume 20μl and then they were frozen on dry ice. After three freeze-thaw cycles, the samples were boiled for 3-5 min. The samples were then analysed by SDS-PAGE (10% gel, running time: 3h at 120V; Table 2.1). The samples were then transferred onto a PVF Immobilon-P membrane (Millipore) using a wet transfer method. The membrane was initially rinsed in methanol followed by a wash in water and was finally soaked in transfer buffer (6.06g Tris, 28.8g glycine, 400 ml of methanol in 2 litres). The transfer apparatus was then setup as followed; 3 3M papers soaked in transfer buffer, the SDS-Page gel, the membrane and 3 more 3M papers. The transfer was run at constant 500mA, maximum volts for 2 hours at 4°C. The membrane was then placed in Block buffer (3% BSA, 1ml/l Twin in PBS) for 1 hour at room temperature. This was followed by the antibody staining at 1:500 concentration in Block solution overnight at 4°C. The membrane was washed 3 times in wash solution (Twin 1ml/l in PBS) for 10 minutes at room temperature. The membrane was then placed in the secondary antibody goat anti-

rabbit IgG HRP (Sigma) at a concentration of 1:3000 in block buffer for 30 minutes at room temperature. The final wash was performed by three washes in wash buffer for 10 minutes. The immunostained bands were detected by chemiluminescence (Pierce).

Table 2.1 10% SDS-PAGE gel

| Separating gel 10% | | Stacking gel 4%* | |
|-----------------------|-------|-----------------------|-------|
| 40% acrylamide: | 24ml | 40% acrylamide: | 3ml |
| 2% Bis-acrylamide: | 13ml | 2% Bis-acrylamide: | 1.5ml |
| 1.5M Tris-HCl pH 8.8: | 25ml | 0.5M Tris-HCl pH 6.8: | 7.5ml |
| 10% SDS: | 1ml | 10% SDS: | 300µl |
| 10% APS (1g/ml): | 500µl | 10% APS (1g/ml): | 150µl |
| TEMED: | 50µl | TEMED: | 30µl |
| Water to 100ml | | Water to 30ml | |

* The stacking gel is applied after the separating gel has solidified.

2.6 Construction of the SRp38-EGFP fusion protein

We have constructed a SRp38-EGFP chimera to follow the behaviour and localisation of SRp38 in living mouse oocytes during early development. The use of GFP tagged proteins bypasses the problem of the artefacts that are caused by the fixation of cells. The human version of SRp38 was amplified by using the pcDNA 3.1 SRp38 vector (provided by James Manley), the 5' primer GGC TCG AGA TGT CCC GCT AC and the 3' primer GGA ATT CGG CCA CTG GAC

TT. The human version of SRp38 is 100% identical in sequence with the equivalent mouse proteins Fusip 1 and NSSR 1. Upon amplification, the insert was subcloned in pEGFP-N1, using the restriction sites XhoI and EcoRI. The SRp38-EGFP vector was expressed in embryos by direct injection into the embryo nucleus. However, mRNA was also produced using the same procedure described for the probe mRNA production. The PCR reaction that was used to incorporate the T7 promoter into the product used the primers for 5' AAA TTA ATA CGA CTC ACT ATA GGG ATG TCC CGC TAC CTG CGT CCC and for 3' GAT CTA GAG TCG CGG CCG CTT TAC.

2.7 Depletion of SRp38 using Morpholino oligonucleotides

In order to study the action of proteins it is often desirable to deplete the specific protein. The most common way to study the action of a specific protein in mouse oocytes is by specific mRNA degradation mediated by double-stranded RNA (dsRNA), which is termed RNA interference (RNAi) (Wianny and Zernicka-Goetz, 2000; Svoboda *et al.*, 2000). In our experiments we used Morpholino antisense oligos (Gene Tools, USA) which have been shown to be both effective and non-toxic for mouse oocytes. An antisense oligo is designed to bind to a complementary sequence in the selected mRNA. This binding prevents the translation of that specific mRNA and, as a consequence, the protein product coded by that particular mRNA is not made. Morpholinos are very stable, specific, non-toxic, water soluble and have long-term activity. Their stability is

due to their structure. Morpholinos are comprised of nucleotides of which the riboses are transformed into morpholines through the introduction of an amine. The bases are, thus, attached to a morpholine instead of a ribose (www.gene-tools.com).

We microinjected specific morpholino oligos to block the expression of both SRp38 in mouse oocytes. The sequence of the SRp38 oligo used was 5' CGG GCT CGG GCA AAC CGT CCG CAG C. Morpholinos are designed to bind to the 5' UTR. The identification of the specific sequence took into account that morpholino oligos work more efficiently when they bind to as much possible of the 5' untranslated region of the target mRNA and when their length is 20-25 bases long. In addition, we made sure that the selected sequence had no self-complementarity to avoid intrastrand pairing. The mouse SRp38 mRNA-specific Morpholino was prepared for us by Gene Tools (OR, USA).

To control for possible non-specific effects of the SRp38 morpholino, a control Morpholino was also purchased. The sequence was CCT CTT ACC TCA TTA CAA TTT ATA. This oligo has no target and no significant biological activity (www.gene-tools.com).

2.8 Immunolocalisation of SRp38 and SC35

Oocytes or embryos were fixed in freshly-prepared 4% paraformaldehyde (in PBS pH 7.4, 1mg/ ml polyvinyl alcohol; PVA) for 20 min. After three washes in PBS/PVA for 5 minutes, the cells were permeabilised in 0.2% Triton X-100 (in

PBS/PVA) for 15 min. The cells were then incubated in blocking buffer (PBS, 2% BSA, 0.2% glycine, 0.02% gelatine) for two hours at room temperature (RT) and then in the rabbit polyclonal anti-human SRp38 antibody (2.5 µg/ml; provided by James Manley) or the mouse monoclonal SC35 (3µg.ml;Sigma) in wash solution at 4°C over night. The cells were then washed 3 times for five minutes in the wash solution described above and incubated with a Alexa conjugated anti-rabbit or anti-mouse secondary antibody (0.5 mg/ml; Alexa Fluor 488 from Molecular Probes) for one hour at RT. This was followed by extensive washing. Included in the first wash was Hoechst (for DNA staining). The immunostaining was visualised using a LSM 510 META confocal microscope. To ensure that comparisons of immunofluorescence could be made between treatment groups or developmental stages, the different samples were scanned and viewed with identical settings. Control oocytes or embryos were also stained by following the same procedure but omitting the stage of the primary antibody.

Analysis of the data obtained by Immunolocalisation was performed using the Metamorph software. Data of average intensity was collected by averaging the pixel intensity of two areas of 20*20 pixels in nuclei and two 50*50 pixel areas in the cytoplasm. The size of speckle was measured using the same software, by selecting the speckles and calculating their size, assuming that an oocyte is 80µm in diameter and an embryo is 90µm in diameter. Size bars are therefore only shown in enlarged images.

2.9 Fluorophores

The two fluorescent proteins EGFP and DsRed Express that are used in the experiments presented in this thesis have mutation compared to the wild type proteins. These mutations are important for the stability, folding, solubility and time of expression of the protein. The mutations of the EGFP compared to the wild type include an insertion of Valine in position 2 of the protein, a Phe-64 to Leu, a Ser-65-Thr and a His-231-Leu (Clontech, UK). DsRed Express also contains a series of mutations which aid in the stability, folding time similar to EGFP compared to the wild type folding time of 24h, enhanced solubility and reduction of tetramer aggregation. The mutations include Arg-2-Ala, Lys-5-Glu, Asn-6-Asp, Thr-21-Ser, His-41-Thr, Asn42-Gln, Val-44-Ala, Cys-117-Ser and Thr-217-Ala. The spectral properties of the two proteins are shown below.

Table 2.2 Spectral properties of EGFP and DsRed Express

| Fluorescent Protein | Quantum Yield | Extinction Coefficient ($M^{-1}cm^{-1}$) | Relative brightness |
|---------------------|---------------|---|---------------------|
| EGFP | 0.70 | 23,000 | 1.00 |
| DsRed Express | 0.90 | 19,000 | 1.06 |

Data obtained from Clontech UK

2.9.2 Fluorescence imaging

The embryos were placed in a drop of HKSOM under oil in a heated chamber (37°C) with a cover slip on the base. The chamber was mounted on a Zeiss Axiovert microscope (Zeiss, Welwyn Garden City, United Kingdom). DsRed Express was excited for 300 msec through a 546/12nm filter using a xenon lamp as a source of light and emission was collected through a 590 nm long pass filter placed in front of a cooled charge-coupled device camera (CoolSnapHQ, Roper Scientific, Tucson, AZ, USA). EGFP was excited for 100 msec through a 480/40 filter and the emission was collected using a 535/50nm filter. Acquisition and emission was controlled by a PC computer running the Metafluor program (Universal Imaging, Downingtown, PA, USA). The blastomere that was expressing the vector was selected and red and green fluorescence levels above the system background were collected for each embryo. The fluorescence of the blastomeres was normally recorded at 1 hour intervals according to each experiment. For the experiments involving the SRp38 overexpression, four areas in the cytoplasm were selected and the average of these four areas was used for analysis.

To ensure that there is no bleed-through of either protein to the other channel, control experiments were performed using the purified recombinant proteins. When different amounts of EGFP were injected in embryos, there was no green bleed-through in the red channel. The fact that there is no bleed through of red

into the green channel can be seen in the experiments of the mutated intron, where no red fluorescence is recorded in the green channel.

2.10 Confocal microscopy

Confocal imaging was preferentially used for immuno-localisation experiments of SRp38, SC35 and for the experiments of live oocytes and embryos expression the SRp38-EGFP.

The advantage of confocal light microscopy is that it can collect the light emitted by a single optical plane of the specimen. A pinhole conjugated to the focal plane obstructs the light coming from objects outside the plane, so that only light from in-focus objects can reach the detector. A laser beam scans the specimen pixel by pixel and line by line. The pixel data are then assembled into an image that is an optical section through the specimen, distinguished by high contrast and high resolution in x, y and z. In order to reduce background noise in the obtained images we used the Kalman averaging function of the confocal microscope. Three successive scans of an image were preferred for Kalman averaging.

For SRp38-EGFP localisation during early development, confocal microscopy was performed using the LSM 510 confocal system.. The system is comprised of a Zeiss Axiovert 200M (equipped with ICS optics and supported by the LSM software) and the LSM laser and scanning module. The oocytes or embryos were placed in a heated chamber (37° C) and SRp38-EGFP was excited using the 488nm line of an argon laser. Fluorescence was collected by a BP 505-530 nm for

emission, collected through a 20x 0.75 NA objective. Laser power was set to 1 or 3% of maximum and images were collected at different intervals depending on the experiment.

An Alexa-conjugated polyclonal anti-rabbit or anti-mouse secondary antibody, and Hoechst were used for detecting the anti-SRp38 antibody, the anti-SC35 antibody and the chromatin respectively, in the cell. The LSM 510 confocal system was used again for imaging of these dyes. For the Alexa-conjugated antibody, confocal images were taken using the 488 nm laser line of an Argon laser and a BP 505-530 nm for emission, using the same set up as SRp38-EGFP imaging. For imaging Hoechst, confocal images were obtained by exciting with the 351 nm laser line of a UV laser and emission was collected through a BP 435-485 nm.

For all confocal imaging experiments a pinhole of 2.22 Airy Units was used, giving a calculated optical slice of 3.5 μ m. The images were analysed using Metamorph software.

2.11 Statistical analysis

All t-tests are two-tailed and based upon two samples (unpaired) with similar variance. Where shown on figures, error bars represent the standard deviation.

Table 2.3 Spectra of Fluorophores used

| <i>Fluorescent Agent</i> | <i>Excitation Maximum (nm)</i> | <i>Emission maximum (nm)</i> |
|--------------------------|--------------------------------|------------------------------|
| Alexa or EGFP | 488 | 507 |
| DsRed Express | 557 | 579 |
| Hoechst | 350 | 450 |

Table 2.4 PCR Reaction Reagents

| <i>Agent</i> | <i>Concentration</i> | <i>Amount</i> |
|-------------------------|----------------------|---------------|
| Template | 20 ng / μ l | 1 μ l |
| Primer Forward/ Reverse | 100 μ M | 0.5 μ l |
| dNTPs | 10 mM | 0.5 μ l |
| 10* Buffer | - | 5 μ l |
| Biology Grade Water | - | 42.5 μ l |
| Taq Polymerase | 5 units / μ l | 0.5 μ l |

Annealing temperature was set at 5°C below melting temperature of the primers used in the reaction. The primers were designed using <http://www.promega.com/biomath/calc11.htm>.

Table 2.5 Concentrations of Injectable reagents

| <i>Agent</i> | <i>Concentration in the pipette</i> |
|--------------------|-------------------------------------|
| Splicing probes | 200 ng / ml |
| U1 Oligonucleotide | 1 mM |
| Morpholinos | 1 mM |

3. Development of a fluorescent probe to measure mRNA splicing in single living cells

3.1 Introduction

Most genes in higher eukaryotes contain non-coding sequences, introns, which need to be excised from the pre-mRNA molecule to generate the mature molecule. This process is called mRNA splicing. The major-class splicing pathway involves five small nuclear ribonucleoprotein particles that recognise consensus sites in the 5' and 3' splice sites of the pre-mRNA molecule. Together with proteins involved in excising and ligating of the pre-mRNA molecule they form a complex called the spliceosome. Excision and ligation of the 5' and 3' splice sites is catalysed by a series of RNA-RNA, RNA-protein and protein-protein interactions. The mature mRNA molecule is then exported to the cytoplasm where translation takes place (Patel and Steitz, 2003) (See section 1.3).

Splicing abnormalities are implicated in disease and are necessary for normal embryonic development. Point mutations in the coding DNA can cause silent or missense mutations. However, when mutations occur in critical splice sites, frameshift and nonsense mutations can completely alter the coding sequence

of the gene. Examples of disease caused by such splicing abnormalities include β -thalassemia, myotonic dystrophy, spinal muscular atrophy, retinitis pigmentosa, atypical cystic fibrosis, Frasier syndrome and even malignancy (Faustino and Cooper, 2003; Garcia-Blanco *et al.*, 2004). Furthermore regulation of splicing is essential for development of many organs and tissues including heart (Cooper, 2005; Ladd *et al.*, 2005), brain (Lins and Borojevic, 2001), kidney (Charlet-Berguerand *et al.*, 2004), even skeletal muscle (Cheema *et al.*, 2005).

mRNA splicing may also play an important role in early mammalian development. Transcription is switched off during the final stages of oocyte maturation and starts again at a specific time after fertilization (late 1-cell – 2-cell stage in the mouse, 4-8-cell stage in the human). This implies that splicing may also be developmentally regulated during this period. Furthermore, if splicing is regulated during this period then the early embryo may prove to be a useful model for investigating the regulation of mRNA splicing and spliceosome assembly. To date no experiments have been performed to investigate the control and regulation of mRNA splicing during the activation of the embryonic genome.

There are two conventional types of assay for splicing efficiency currently in use. Firstly, assays are performed by transfection of gene fragments to mammalian cells. The resultant mRNA molecules are analysed by means of RT-PCR. The sizes of the RT-PCR fragments allow the determination of spliced or unspliced product. The splicing efficiency can be calculated as the ratio of spliced : unspliced mRNA (Lytle and Steitz, 2004). Secondly, *in vitro* assays are

performed by adding radioactively labelled mRNA transcripts to nuclear extracts. The intermediate stages and products of splicing are then visualised by urea-PAGE electrophoresis and autoradiography (Mayeda and Krainer, 1999). A ratio of spliced and unspliced mRNA can then be calculated from the intensities of spliced and unspliced mRNAs. These methods are reliable and straight forward, but lack the ability to monitor splicing in single cells or in a dynamic system, such as development.

Two different approaches have been reported recently that allowed the measurement of splicing activity in live cells. This was firstly demonstrated by construction of an EGFP minigene. This minigene contained an intron that intervenes the EGFP reading frame. Cell lines were then transfected with the EGFP construct. Splicing activity would then be determined by fluorescence of the EGFP protein, whereas splicing inhibition would lead to expression of a truncated protein and therefore no fluorescence (Sazani *et al.*, 2003; Wang *et al.*, 2004). However, such a system does not provide an internal reference that would yield internal information of transcription level or transfection efficiency.

In 2002, Nasim and colleagues described an elegant approach to monitor splicing activity in cultured mammalian cells. The group constructed a reporter vector containing the reading frames of β -galactosidase and luciferase, separated by an artificial intron. This intron comprised an exon-intron (5' splice site) sequence donated from adenovirus, three in frame stop codons, and an intron-exon (3' splice site) sequence donated from the human tropomyosin TPM3 gene.

All transfected cells express the β -galactosidase gene. The enzymatic activity of β -galactosidase indicates reference transcription and translation levels of the reporter. However, only cell lines in which splicing has taken place express the luciferase gene. In the absence of splicing, the translation is terminated at the stop codon, present between the 5' and 3' splice site of the reporter. Splicing efficiency was then calculated as a ratio of the enzymatic activities between the two enzymes. This assay allows splicing efficiency to be determined in live mammalian cells, but it unfortunately requires the destruction of the cells and for the measurement to be performed on populations of cells rather than single cells.

In order to study the mRNA splicing in single living mammalian embryos, we set out to design a fluorescent probe for mRNA splicing. To construct such a reporter, we substituted the two enzymes in the reporter described earlier (Nasim *et al.*, 2002) with two fluorescent proteins, DsRed Express and EGFP.

In this chapter I describe the preparation and characterisation of a fluorescent splicing sensor. Here we show that the splicing sensor can be used to monitor splicing activity in single living cells. We have used this probe to show that interfering with splice site during activation of the embryonic genome results in inhibition of embryo development at the 2-cell stage.

3.2 Results

3.2.1 Characterisation of the probe

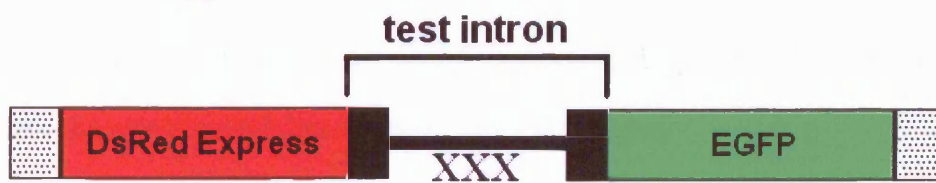
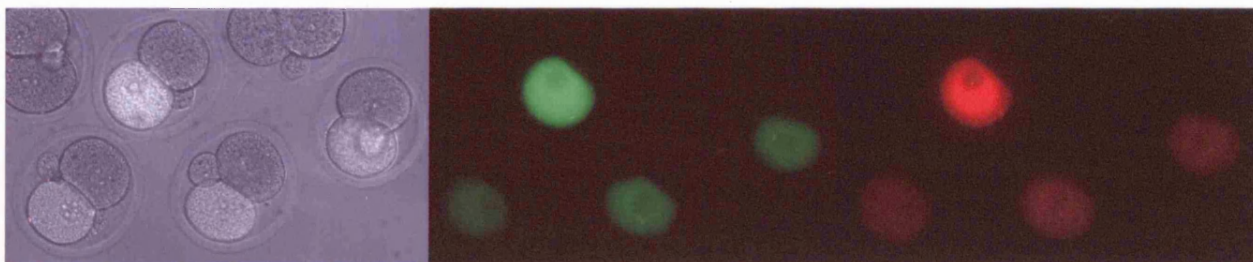
The production of the probe was achieved by a series of sub-cloning experiments outlined in section 2. The general principle of how the probe operates is shown in Figure 3.1A. DsRed Express (RFP) is located downstream of a CMV promoter that drives transcription in the nucleus of the early embryos. The intron encompasses three in frame translational stop codons. In the absence of splicing, translation ends after RFP, thereby providing fluorescence exclusively from RFP. In the presence of splicing the translational machinery will read through to the EGFP, thereby providing fluorescence from RFP and EGFP.

To test the principle, we constructed a vector, DsRed Express-TN24-EGFP, by cloning EGFP into the multiple cloning site of DsRed Express and then inserted the test intron from plasmid pTN24 (Nasim *et al.*, 2002). The TN24 intron contains a 5' exon-intron sequence from adenovirus and a 3' intron-exon sequence from the tropomyosin TPM3 gene. To ensure that both fluorescent proteins are only apparent when splicing has taken place, the EGFP sequence in the complete construct is out of frame with the upstream RFP reading frame. Therefore, EGFP becomes in frame with the upstream coding sequence, only after splicing has taken place.

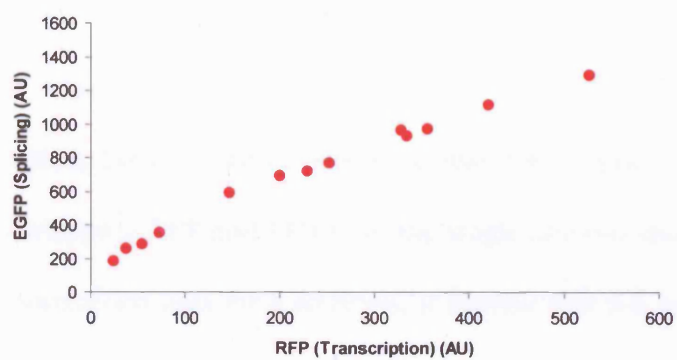
The probe was tested by microinjection of the DNA into the nuclei of single blastomeres of 2-cell embryos. The embryos were cultured for 8 hours and then examined using appropriate filter sets and epi-fluorescent microscopy

(Figure 3.1B). The probe is expressed in the injected blastomere. The relative levels of splicing (EGFP intensity) as a function of transcription (RFP intensity) 8 hours after injection are plotted in Figure 3.1C. Each point represents an individual embryo. If the reporter assay compensated for variation in the level of expression, it would be predicted that the intensity of EGFP fluorescence (spliced mRNA) would be proportional to the intensity of RFP fluorescence (total expressed mRNA). The data in figure 3.1C fits these results. The most probable explanation of the different levels of transcription may reflect variations in the amount of DNA injected and different transcriptional activities in individual embryos.

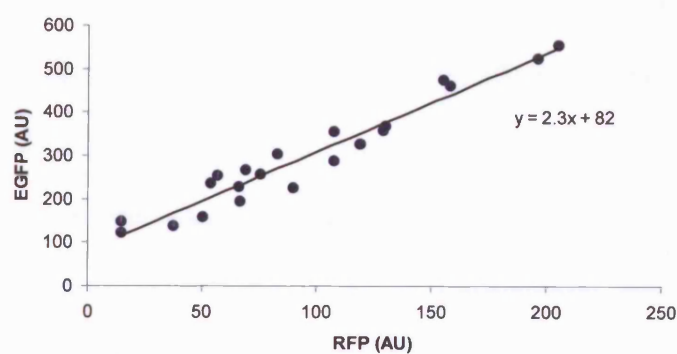
The data show that the fluorescence detected as a result of transcription (RFP) is markedly less than the fluorescence obtained from spliced product (EGFP). For example, fluorescence as a result of transcription of RFP is at 100 AU correlates with a value of spliced product EGFP of 300 AU. Even if 100% of the mRNA were spliced, the number of molecules of EGFP should not exceed that of RFP. In our experimental conditions we found that RFP fluorescence intensity was ~2-3 fold lower than EGFP fluorescence, despite a 3-fold large acquisition time for RFP. To determine whether this was a property of relative fluorescent intensities of EGFP and RFP or if it was a property of the probe, a standard curve was prepared by injecting equal amounts of recombinant purified EGFP and RFP across a range of concentrations (Figure 3.1D). This clearly shows that for the

A**B****C**

Splicing efficiency of DsRed Ex - TN24 - EGFP

**D**

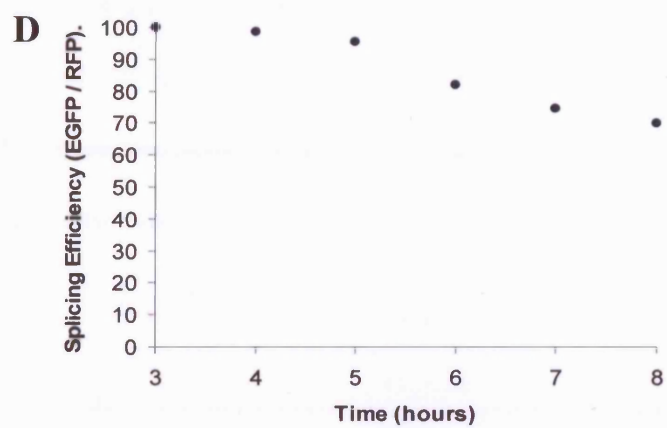
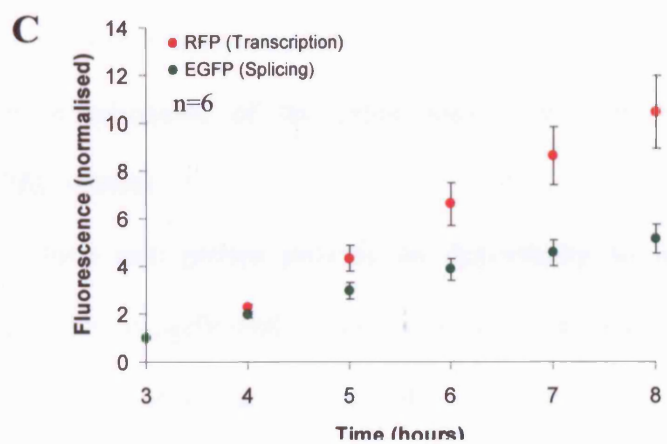
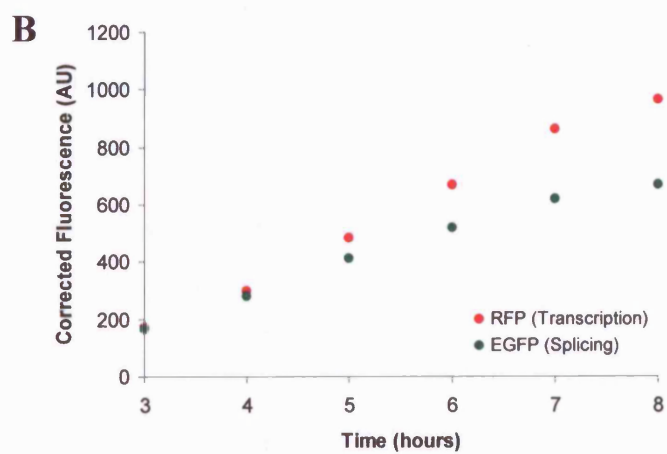
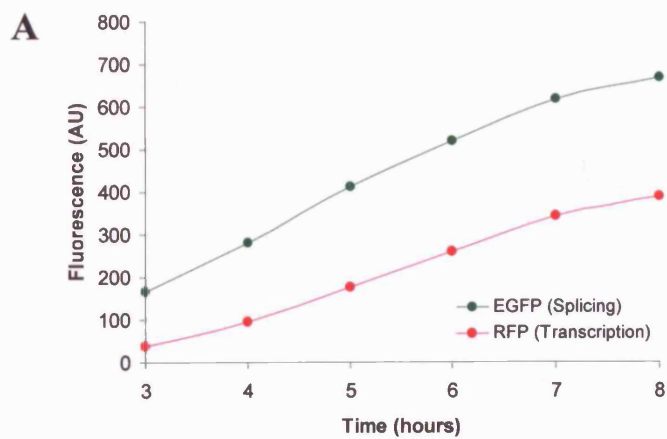
Fluorescence of equal amounts of RFP and EGFP



same concentration of protein, EGFP is brighter than RFP (Clontech), consistent with the experimental data obtained using this probe.

3.2.2 Monitoring the time course of expression and splicing

To monitor transcription and splicing, we injected the reporter and monitored fluorescence every hour from 3 hours after injection. The increase in RFP and EGFP fluorescence with time shows that transcription and splicing continue over the 8 hour time period (Figure 3.2A). Using the standard curve shown in Figure 1D, the RFP fluorescence values have been corrected so as to allow splicing efficiency to be compared more readily across a population of embryos. This corrected value was obtained by introducing the RFP value into the equation $y=2.3x+82$. This correction is applied to all RFP data presented from this point on. After correction, the expectation would be that 100% splicing efficiency would result in overlapping RFP and EGFP. In the single embryo shown in Figure 3.2B and in the normalised data for 6 embryos, it is clear that 5-6 hours after injection of the plasmid the levels of RFP and EGFP proteins diverge. The relative decline in EGFP intensity suggests that the rate of splicing does not continue to match transcription. By plotting the apparent splicing efficiency (EGFP (Spliced) / RFP (Transcribed)) a gradual decrease is seen such that it is approximately 70% by 8 hours after injection. One possible explanation for this gradual decrease in splicing efficiency is that the level of transcription saturates the splicing machinery.



3.2.3 The probe works using different intronic sequences

The intron used to date, TN24, is a construct using an exon–intron sequence from Adenovirus and an intron-exon sequence from tropomyocin (Nasim *et al.*, 2002). To test whether other introns function in a similar manner, we produced a vector containing an exon-intron-exon sequence from IgM gene (M1M2). A second vector was generated that has the same intron, but it lacks a downstream enhancer sequence (M1M2ΔE) (Liu *et al.*, 1998; Liu *et al.*, 2000; Nasim *et al.*, 2002) (Figure 3.3A). Both these vectors include a single stop codon. Similar to the TN24 vector, the EGFP is out of frame with the upstream sequence of the DsRed Express in both M1M2 vectors. After microinjection of the vectors into a blastomere of a 2-cell embryo the overall pattern of expression and splicing activity for both vectors over 8 hours is similar to that seen for TN24. Figure 3.3A presents a schematic of the probe expression outcome of the M1M2 and M1M2ΔE vectors.

These two probes provide an opportunity to investigate whether the splicing probe is sufficiently sensitive to detect differences between M1M2 and the M1M2ΔE. The absence of the enhancer sequence in M1M2ΔE predicts it will be spliced less efficiently than M1M2. This was tested by examining the levels of splicing in 2-cell embryos 8 hours after microinjection of the vectors. In all the embryos examined across a range of different levels of transcription, the level of splicing is less in the M1M2ΔE intron than M1M2. This data shows that the

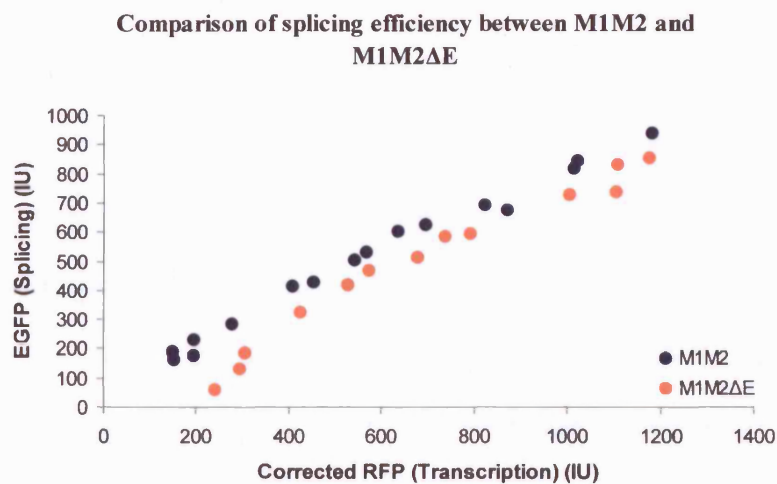


Figure 3.3. The effect of an enhancer sequence on splicing. The two probes were injected and the embryos were monitored after 8 hours of culture. The graph shows the corrected fluorescence levels of the two probes. The enhancer-less intron (M1M2ΔE) is shown to be spliced less efficiently than the intact intron (M1M2).

fluorescent strategy to monitor splicing can be used to detect differences in splicing efficiency.

3.2.4 Splicing is necessary to achieve expression of the EGFP

Since the three vectors described above presented similar splicing patterns, we have performed further experiments with the TN24 vector to ensure that the EGFP fluorescence only appears in conditions where splicing is expected.

Firstly, we have attempted to inhibit all splicing by removing the 5' splice site from the vector. As a result we predict that RFP should be produced in the absence of any EGFP (Figure 3.4A). Microinjection of this vector revealed that after 8 hours of culture, no splicing activity was evident over a wide range of transcription levels (Figure 3.4A). This confirms that the 5' splice site is essential for splicing of the mRNA and that the probe only yields EGFP when splicing takes place. As a second test that EGFP fluorescence was dependent on splicing, we in vitro transcribed mRNA of the probe. The mRNA was microinjected into the cytoplasm of 2-cell embryos and splicing activity was monitored. Since splicing takes place in the nucleus, we hypothesised that the injected mRNA would be translated with no evidence of splicing. As predicted, we detected RFP, indicating efficient transcription and translation but no EGFP (Figure 3.4B). These experiments show that the splicing probe provides a reliable measure of mRNA splicing activity in single living cells.

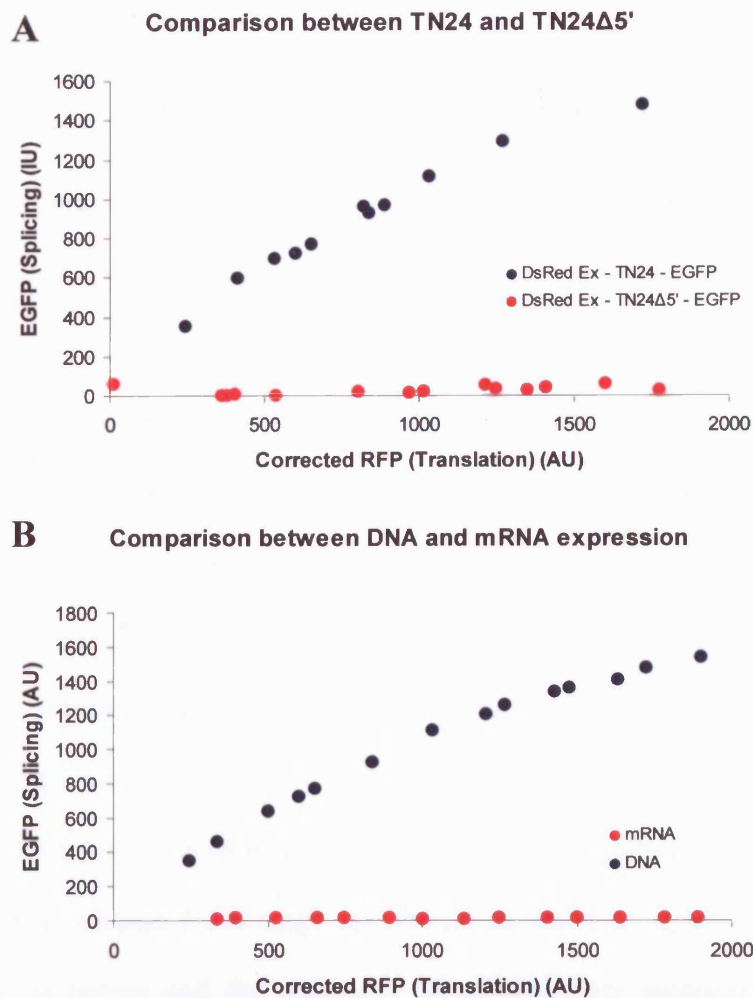


Figure 3.4. A. Comparison of splicing efficiencies between the vectors comprising of the TN24 and the TN24 Δ 5' (mutated) introns, in single blastomeres 8 hours after injection. The vector comprising the TN24 intron is spliced as described earlier. No splicing is taking place in the TN24 Δ 5' vector with the mutated intron. 5' splice site is essential for the splicing process. This result also indicates that the stop codons are functioning. **B.** Comparison between splicing efficiencies of the DNA injected in the nucleus of an embryo and the same mRNA sequence injected in the cytoplasm of an embryo. DNA in the nucleus is spliced as expected, yielding the fusion protein DsRed Express-EGFP. The mRNA injected in the cytoplasm yields only RFP showing normal translation but no splicing activity in the cytoplasm.

3.2.5 Inhibition of a major snRNP, U1, inhibits splicing in vivo

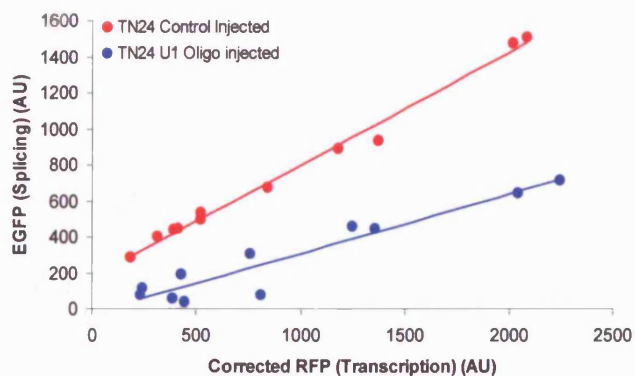
Upon confirmation that the EGFP fluorescence could only be detected in conditions that supported mRNA splicing, we set out to test whether we could inhibit splicing in vivo and examine the effects on embryo development.

In splicing assays in vitro, splicing can be inhibited using an oligonucleotide complementary to the 5' end of the U1 snRNA (Kramer *et al.*, 1984). This portion of the U1 snRNA recognises the 5' splice site by base pairing complementarity. U1 snRNA was therefore an obvious target for inhibition of the major-class pathway of splicing and provided an opportunity to address the role of splicing in embryo development.

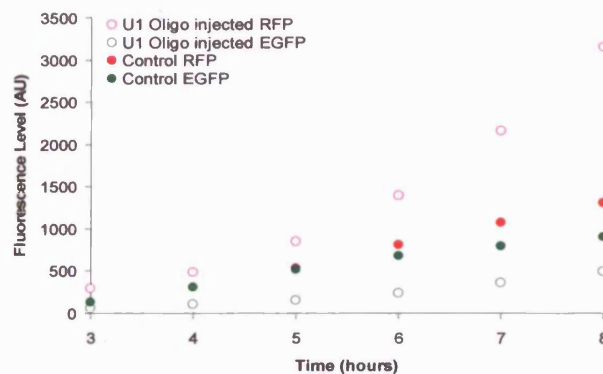
The oligo complimentary to U1 snRNA was injected 45h post hCG, 3 hours prior to the mid 2-cell stage, and the probe was then injected in the mid 2-cell stage as before and the efficiency of splicing was monitored. In control embryos injected with scrambled oligo, the typical pattern of RFP and EGFP was detected showing normal transcription and splicing. In embryos injected with the U1 oligo 8 hours previously there was a measurable decrease in splicing across a wide range of expression levels, using the TN24 (Figure 3.5A), the M1M2 (Figure 3.5C) and the M1M2ΔE (Figure 3.5E). For example, when RFP fluorescence is 1000AU, the oligo complementary to U1 reduced EGFP to 20-40% of the control (Table 3.1).

Fluorescence records for the time course of transcription and splicing from 3 hours is shown for representative single embryos in Figures 3.5 B, D, and F. In

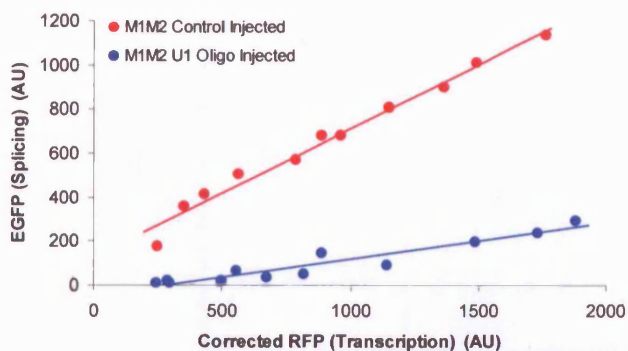
A Effect of U1 Oligo in Splicing Efficiency using TN24



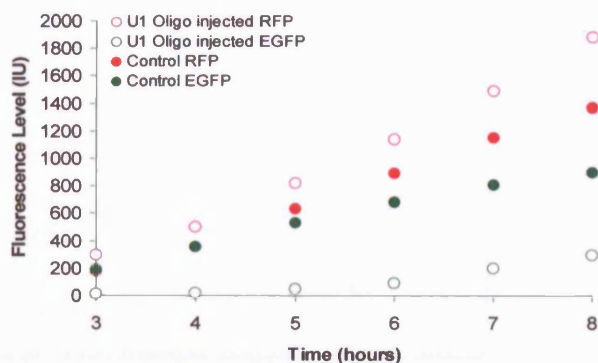
B Representative of Control and U1 oligo injected embryos using TN24 over a period of 8 hours



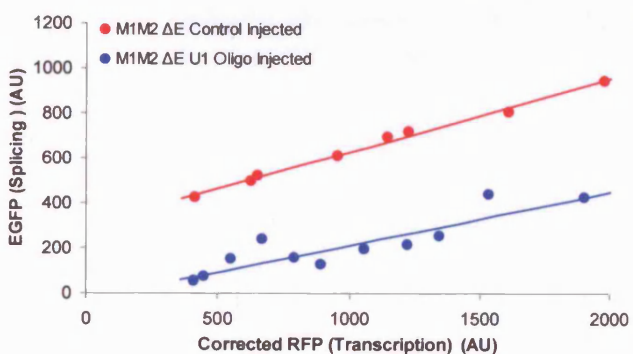
C Effect of U1 Oligo on Splicing Efficiency using M1M2



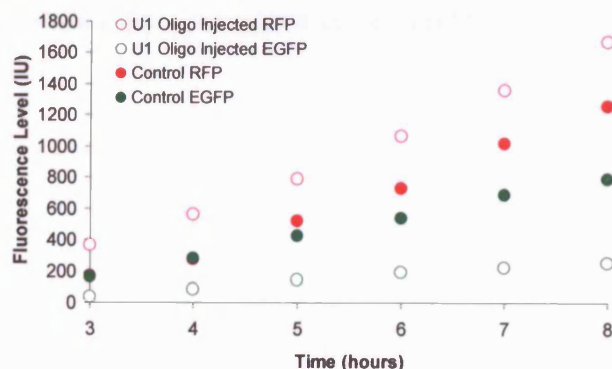
D Representative Control and U1 Oligo injected embryos using the M1M2 over a period of 8 hours



E Effect of U1 Oligo on Splicing Efficiency using M1M2ΔE



F Representative Control and U1 injected embryos using M1M2ΔE over a period of 8 hours



| Vector | | EGFP fluorescence | Relative Splicing Efficiency |
|--------|------------------|----------------------|---------------------------------|
| TN24 | U1 Oligo | 307 | 38 % |
| | Control Oligo | 799 | |
| M1M2 | U1 Oligo | 118 | 17% |
| | Control Oligo | 709 | |
| M1M2ΔE | U1 Oligo | 209 | 33% |
| | Control Oligo | 625 | |

Table 3.1. The table demonstrates the decrease in splicing efficiency of the U1 oligo injected embryos compared to the controls. The average decrease in splicing efficiency was between ~60% and ~80% compared to the control splicing efficiency. The EGFP fluorescence values presented correspond to a level of RFP of 1000 AUs.

all cases the U1 injected embryos show a decline in green fluorescence, consistent with inhibition of splicing. Interestingly, in each case the RFP fluorescence increased in U1 oligo injected embryos compared to controls. The shift of red may reflect an increase in export of unspliced transcripts to the cytoplasm for translation in the absence of U1 snRNP binding activity.

3.2.6 Inhibition of U1 dependant splicing prevents development beyond the 2-cell stage

Transcription necessary for subsequent development first occurs at the mid 2-cell stage. To date no experiments have investigated whether interfering with the mRNA splicing impacts on the ability to progress beyond the 2-cell stage. To test this hypothesis that splicing is essential for development beyond the 2-cell stage, we have injected the U1 oligo, known to block splicing, and monitored development. The results clearly show that U1 injected but not scrambled oligo injected embryos all block at the 2-cell stage of development (Figure 3.6). This indicates a critical role for transcription and splicing at the time of embryonic genome activation.

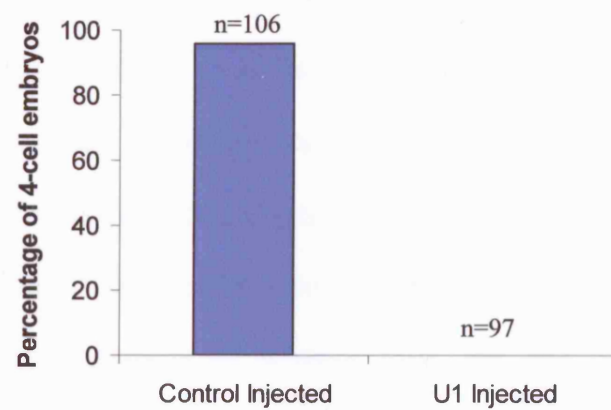


Figure 3.6. Effect of U1 oligo injection on development of embryos. Control injected embryos develop normally to the 4-cell stage, whereas U1 oligo injected embryos arrest at the 2-cell stage.

3.3 Conclusions

This chapter presents a novel, fluorescent based probe for monitoring splicing activity in single cells. The disadvantages of the conventional splicing assays are that they are biochemical, destructive assays that do not allow the measurement of splicing efficiency in a single cell basis in early development. It was demonstrated that fluorescent proteins such as DsRed Express and EGFP can mature as quickly as three hours after injection of our probes and report splicing efficiency. The probes are designed in such a way so that both fluorescent proteins are expressed only if splicing has taken place. Two measures are used to ensure that only RFP is expressed in the absence of splicing. First, the stop codons in the intronic sequence downstream of RFP and second that the downstream EGFP is out of frame in the complete cDNA.

The ability of the probe to detect changes in splicing efficiency was challenged by inhibition of splicing by the means of a U1 oligonucleotide injected before injection of the probe. There was a great reduction in the splicing efficiency of more than 60% in all cases after injection of the U1 oligo. The splicing properties of TN24 were also challenged by mutation of the 5' splice site and injection of mRNA in the cytoplasm, which led to the exclusive production of RFP and therefore no splicing. The three different probes that were used demonstrated that splicing efficiency is highly dependant on the 5' and 3' splice site sequences and also on the presence or not of an exonic splicing enhancer sequence.

These probes demonstrated for the first time the monitoring of splicing activity in live cells during a short time course. It was shown that splicing efficiency could be monitored as soon as 3 hours after injection of the DNA. Overloading of the cell with probe transcripts and embryonic transcripts may explain the decrease in splicing efficiency 5 hours after injection. Transcription was unaffected during this period of 8 hours, as indicated by the linear increase of RFP. This suggests that the rate of splicing and transcription can be uncoupled and that there is limited feedback from splicing machinery to control the rate of transcription. These results uncover a limitation of this system. The fact that plasmid DNA is injected in the nucleus results in continuous production of probe transcripts, which may lead to the proposed saturation of splicing machinery.

Some considerations were raised during the construction of these probes. One of them was that unspliced mRNA tends to remain in the nucleus, because splicing factors that interact with the pre-mRNA prevent its transport to the cytoplasm (Legrain and Rosbash, 1989). Such an effect would be disastrous for our assay, because if only spliced mRNA was expressed, it would be impossible to measure splicing efficiency. However, our results, results by Nasim and colleagues (2002) and results from Legrain and Rosbash (1989) show that unspliced mRNA is exported and translated. The Legrain and Rosebash study demonstrated that a mutation in the U1 snRNA decreased splicing efficiency and caused a large increase in translation of their minigene. Our experimental approach actually mimics the inhibition of U1 snRNA binding to the pre-mRNA,

in a similar way to that presented by Legrain and Rosebash (1989). In our experiments the U1 oligo binds to the U1 snRNA, therefore inhibiting its binding to the pre-mRNA. The fact that RFP fluorescence was increased in U1 oligo injected embryos compared to controls, suggests that when U1 snRNP does not bind to the pre-mRNA, there may be an increase in mRNA export, as shown by Legrain and Rosebash. Our results also suggest that the amount of unspliced mRNA that is exported from the nucleus does not depend on splicing efficiency; otherwise large variations would be recorded in the levels of red fluorescence with similar amounts of the probe being injected.

A second consideration was nonsense mediated decay (NMD). NMD is the decay of mRNA containing a premature stop codon after splicing has taken place. The mechanism of NMD compromises mRNA that appears to contain a termination codon ~25 nucleotides upstream of a splicing exon-exon junction. NMD therefore provides a defence mechanism to the cell from truncated and possibly deleterious proteins (Lejeune and Maquat, 2005). However, NMD occurs only in spliced and not unspliced mRNA. Therefore NMD would not actually occur in our termination codon if proper splicing has taken place, and such a process would not interfere with our results.

Development was affected by the inhibition of splicing by compromising the activity of a major snRNP, U1. All 2-cell embryos injected with the U1 oligonucleotide arrested at the 2-cell stage. The zygotic genome activation occurs in the mouse in two phases; in late 1-cell stage and at the second half of the 2-cell

stage. Up until this stage all mRNAs required for development have been processed during embryogenesis (Latham, 1999) and their expression is regulated by polyadenylation (Richter and Theurkauf, 2001). After zygotic genome activation, transcription of embryonic messages requires splicing for proper protein expression. Our experiments verified that inhibition of splicing in that stage results in arrest of development, due to expression of truncated and deleterious proteins in the absence of splicing.

More probes can be designed in such a way that the splicing efficiency of different intron sequences can be investigated. An example would be to use introns that would be spliced by minor class pathway, and determine if, when and possibly how these two distinct pathways are important in early development.

These probes possess a great potential for quick and easy results regarding the measurement of splicing efficiencies. These properties allow these probes to be very useful in experiments performed in early development, where cell divisions occur during short periods of time, due to the quick and easy way of obtaining results.

4. The development of nuclear “speckles” in early mouse development

4.1 Introduction

Nuclear “speckles” are structures that contain snRNPs and SR proteins. These structures are otherwise known as interchromatin granule clusters (IGCs), which appear to be important in spliceosome assembly and also act as storage sites for factors essential for splicing (Lamond and Spector, 2003). One of the splicing factors localising in the nuclear speckles is SC35.

SC35 is a protein of 35kDa that belongs to the SR protein family. SR proteins are serine-arginine rich proteins or otherwise known as non snRNPs and they play a numerous roles in spliceosome assembly and regulation of the splice site selection / alternative splicing (refer to section 1.5). SR proteins possess one or two N-terminal RNA binding domains (one in the case of SC35) and a C-terminal domain rich in serine-arginine (SR domain), which functions as a protein interaction domain (Graveley, 2000). In transcriptionally active cells, SC35 localises in the nucleus in structures called nuclear speckles, where most splicing factors are localised. The RS domain of SC35 is necessary and sufficient as a targeting signal to localise SC35 to the speckles (Caceres *et al.*, 1997). Many SR proteins such as SRp20, 9G8 and ASF/SF2 shuttle between the nucleus and the

cytoplasm (Caceres *et al.*, 1998), but SC35 is retained in the nucleus due to a strong nuclear retention signal on its RS domain (Cazalla *et al.*, 2002).

A role for SC35 in splicing was first discovered by depleting it from nuclear extracts. SC35 was also shown to co-localise with snRNPs (Fu and Maniatis, 1990) and RNA Polymerase II (Bregman *et al.*, 1995). SC35 is required for spliceosome assembly (Fu and Maniatis, 1990) and it was later shown that SC35 interacts with both U1 snRNP, which binds to the 5' splice site, and the U2AF and U2 snRNP, which bind to the 3' splice site (Fu and Maniatis, 1992; Bruzik, 1996). SC35 can activate splicing in vitro in S100 HeLa cell extracts (Fu *et al.*, 1992), or compensate for the loss of the U1 snRNP in vitro (Tarn and Steitz, 1994). Furthermore it can reconstitute splicing in U2AF₆₅ depleted nuclear extracts but only in the presence of U1 (Tarn and Steitz, 1995). This suggests that the SC35 is involved in the selection of 5' and 3' splice sites and that there are distinguishable mechanisms for the binding of U2 in the pre-mRNA depending on whether U2AF is present or not (MacMillan *et al.*, 1997).

SR proteins are generally regulated by phosphorylation. It has been shown in nematodes that, in their inactive form they are hyperphosphorylated and localise in the cytoplasm of the cell. After zygotic genome activation, they become active by partial dephosphorylation and localise to the nucleus (Sanford and Bruzik, 1999; Sanford and Bruzik, 2001).

SC35 is also implicated in alternative splicing. It was shown that SC35 can favour proximal over distal 5' splice site selection and also influence the 3' splice

site selection by favouring the proximal 3' splice site (Fu *et al.*, 1992). SC35 was also shown to have antagonistic effects on SF2/ASF, by inhibiting the splicing of a particular exon, in which the splicing was enhanced by SF2/ASF (Gallego *et al.*, 1997). Later experiments showed that SC35 enhanced splicing when an exonic splicing enhancer sequence was present in the downstream exon (Liu *et al.*, 2000). This substrate specificity was shown to be mediated by the RRM domain of SC35. This was demonstrated in studies where the RRM domains of SF2/ASF and SC35 were swapped (Chandler *et al.*, 1997; Mayeda *et al.*, 1999).

SC35 was shown to be induced in cases of human ovarian cancer (Fischer *et al.*, 2004) and in HIV infected cells (Maldarelli *et al.*, 1998) and was implicated in alternative splicing in both cases. Knock-out mice with a deletion of the SC35 gene from the thymus, caused a dramatic decrease in the size of thymus and also caused the alternative splicing (lack of exon 5 from mRNA) of the CD45 marker, which led to severe block in T-cell development (Wang *et al.*, 2001). SC35 has been implicated in a mental retardation cases caused by lactic acidosis. A mutation downstream of exon 7 of E1-alpha dehydrogenase, caused SC35 to recognise it as a cryptic splice site, causing aberrant splicing of the gene (Gabut *et al.*, 2005). Finally, the level of SC35 expression is autoregulated by alternative splicing, by an exon inclusion and intron excision in the 3' untranslated mRNA region under overexpression conditions. The resulting mRNA is unstable thereby causing the reduction of newly synthesised SC35 (Sureau *et al.*, 2001).

SC35 has been shown to co-localise with a nuclear pool of PIP₂, implicating PIP₂ in regulation of splicing and spliceosome assembly (Osborne *et al.*, 2001). PIP₂ is a membrane bound phospholipid found in all eukaryotic cells, mainly in the plasma membrane. PIP₂ is involved in many cellular processes including the release of Ca²⁺ (Berridge, 1997) and the regulation of the actin cytoskeleton (Halet *et al.*, 2003). The co-localisation of PIP₂ with SC35 demonstrated by Osbourne and colleagues suggests that PIP₂ may play a role in pre-mRNA splicing. PIP₂ was found to co-localise with SC35 in a speckled appearance within the nucleus of the cell (Osborne *et al.*, 2001). In the same study, PIP₂ depleted nuclear extracts showed an 80% decrease in splicing efficiency, demonstrating a new role of PIP₂ in the cell. However, the regulation of mRNA processing during early development is not yet clear. Zygotic genome activation in the mouse occurs at the second half of the 2-cell stage. Therefore, this transition from maternal dependence of the embryo to zygotic dependence provides a transition in which the importance of SC35 and PIP₂ during early development and splicing may be investigated.

In this study, I have used SC35 and PIP₂ localisation in the nucleus to investigate the evolution of nuclear speckles during oocyte maturation and early embryo development.

4.2 Results

4.2.1 SC35 localisation during oocyte maturation using immunofluorescence

Immunofluorescence techniques were used in order to investigate the localisation of SC35 during early mouse development. To ensure that the immunofluorescent images can be compared, the staining of all oocytes and embryos in any one experiment was performed at the same time using identical conditions. The localisation of SC35 was investigated in oocytes obtained from pre-antral follicles through to embryos at the 8-cell stage. In preantral follicles, SC35 localises in the nucleus in multiple small speckles (Figure 4.1 A and Ai). When fully grown GV-stage oocytes were stained immediately after collection, small speckles were seen within the germinal vesicle $1.4 \pm 0.25 \mu\text{m}$ ($n=6$), both when collected in IBMX free or enriched media (Figure 4.1 B and C respectively). GV-stage oocytes that were cumulus-free upon release from the follicle appeared with enlarged speckles of $2.7 \pm 0.8 \mu\text{m}$ in diameter (Figure 4.1 D). When the oocytes were fixed and labelled 1 hour after collection, the speckle size was larger, $3.34 \pm 0.75 \mu\text{m}$ in diameter ($n=6$) (Figure 4.1 E). It was also surprising to see that immature oocytes arrested in IBMX for the same period of time also presented staining in the nucleus in the form of large speckles, $3.26 \pm 0.89 \mu\text{m}$ in diameter ($n=6$) (Figure 4.1 F). After GVBD, SC35 redistributed to the cytoplasm (Figure 4.1 G), where it remained for the duration of oocyte maturation. Finally, in the MII stage, a stage in which the cell does not possess a nucleus, SC35 localises in the cytoplasm of the cell (Figure 4.1 H). In

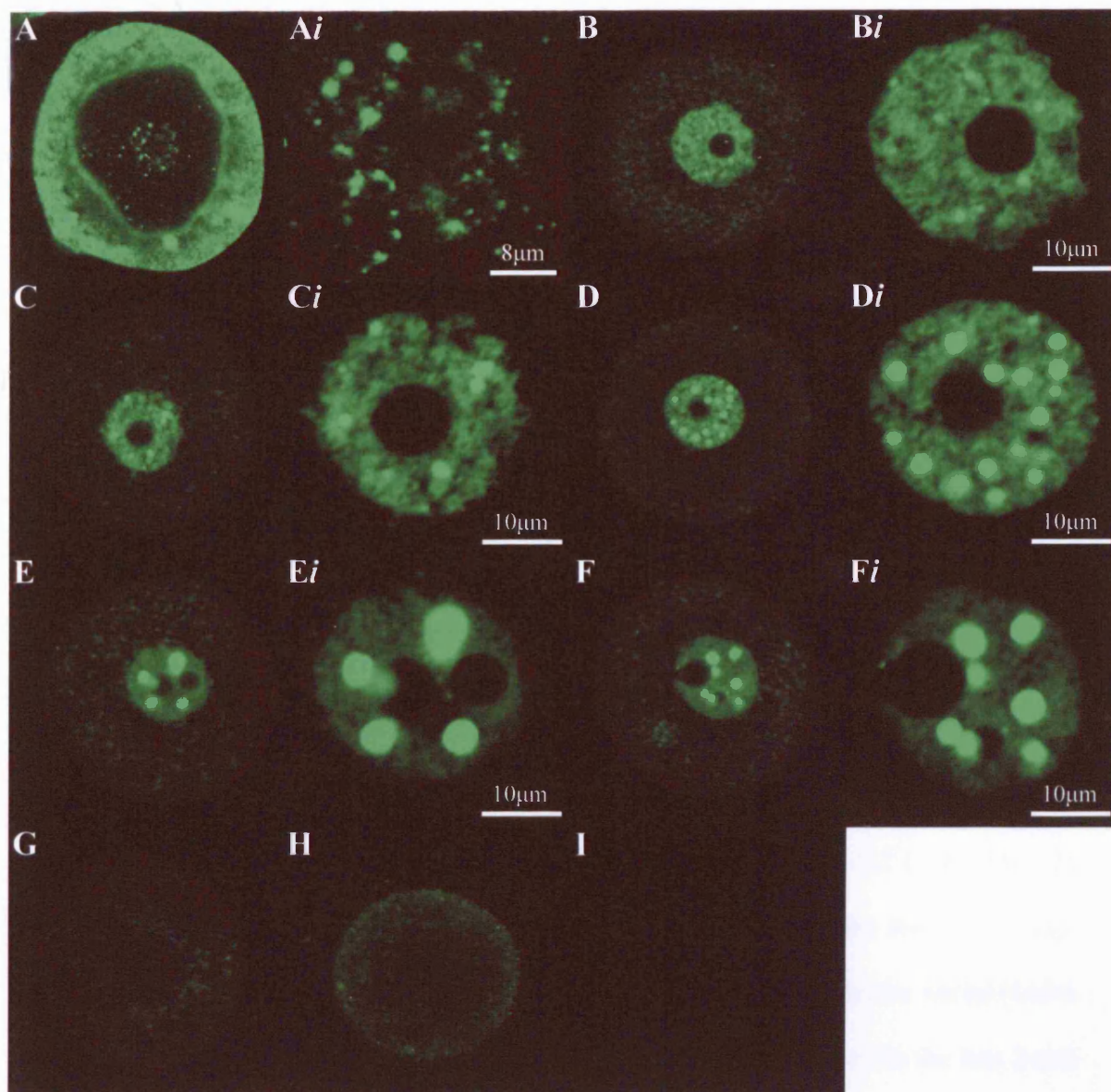


Figure 4.1. The localisation of SC35 during oocyte maturation. In all images *i* corresponds to magnified image of the nucleus. **A.** Pre-antral follicle. SC35 localises in the nucleus in small speckles. **B.** GV oocyte fixed in IBMX free medium. Speckles of $1.4\mu\text{m}$ in diameter are seen within the GV. **C.** An oocyte collected in medium containing IBMX and fixed immediately after isolation appears with similar speckles as in GV oocyte collected in IBMX free medium. **D.** An oocyte that was cumulus free on release from the follicle. The oocyte appears with larger speckles, $2.7\mu\text{m}$ in diameter. **E.** GV oocyte released for 1 hour. Speckles are enlarged to $3.34\mu\text{m}$ in diameter. **F.** GV oocyte arrested in IBMX for 1 hour. Surprisingly enlarged speckles $3.26\mu\text{m}$ in diameter are seen. **G.** After germinal vesicle breakdown speckles disappear and SC35 distributes to the cytoplasm. **H.** MII. SC35 is distributed in the cytoplasm. **I.** A control oocyte, stained only with the secondary fluorescent antibody.

control GV-stage oocytes (Figure 4.1 I), there was no staining in the nucleus or the cytoplasm.

4.2.2 Localisation of SC35 during early embryo development

Immunofluorescence of SC35 in early embryos is shown in figure 4.2. Differences observed in the nuclear-cytoplasmic ratios during this period can be seen in figure 4.3. At pronucleus stage (Figure 4.2 A) (26h post hCG) SC35 remains in the cytoplasm of the cell. Prior to NEBD (30h post hCG), SC35 is recruited to the pronuclei of the embryo. This is accompanied by an apparent decrease in immunoreactivity in the cytoplasm (Figure 4.2 B) and an increase in nuclear-cytoplasmic ratio (Figure 4.3). At the early and mid 2-cell stage, maternal SC35 remained localised in the nuclei of the embryo (Figure 4.2 C and D). The first signs of arrangement of the SC35 in speckles is seen in the mid 2-cell stage (Figure 4.2 D). An increase in the concentration of SC35 both in the nuclei (165% increase, n=6) and the cytoplasm (~400% increase, n=6) is seen in the late 2-cell embryo (Figure 4.2 E), compared to the mid 2-cell stage (Figure 4.2 D). The speckle size at the late 2-cell stage is $1.45 \pm 0.2 \mu\text{m}$. Finally, in 4-cell and 8-cell stage embryos the appearance of SC35 localisation resembles that of the late 2-cell stage. (Figure 4.2 F and G respectively).

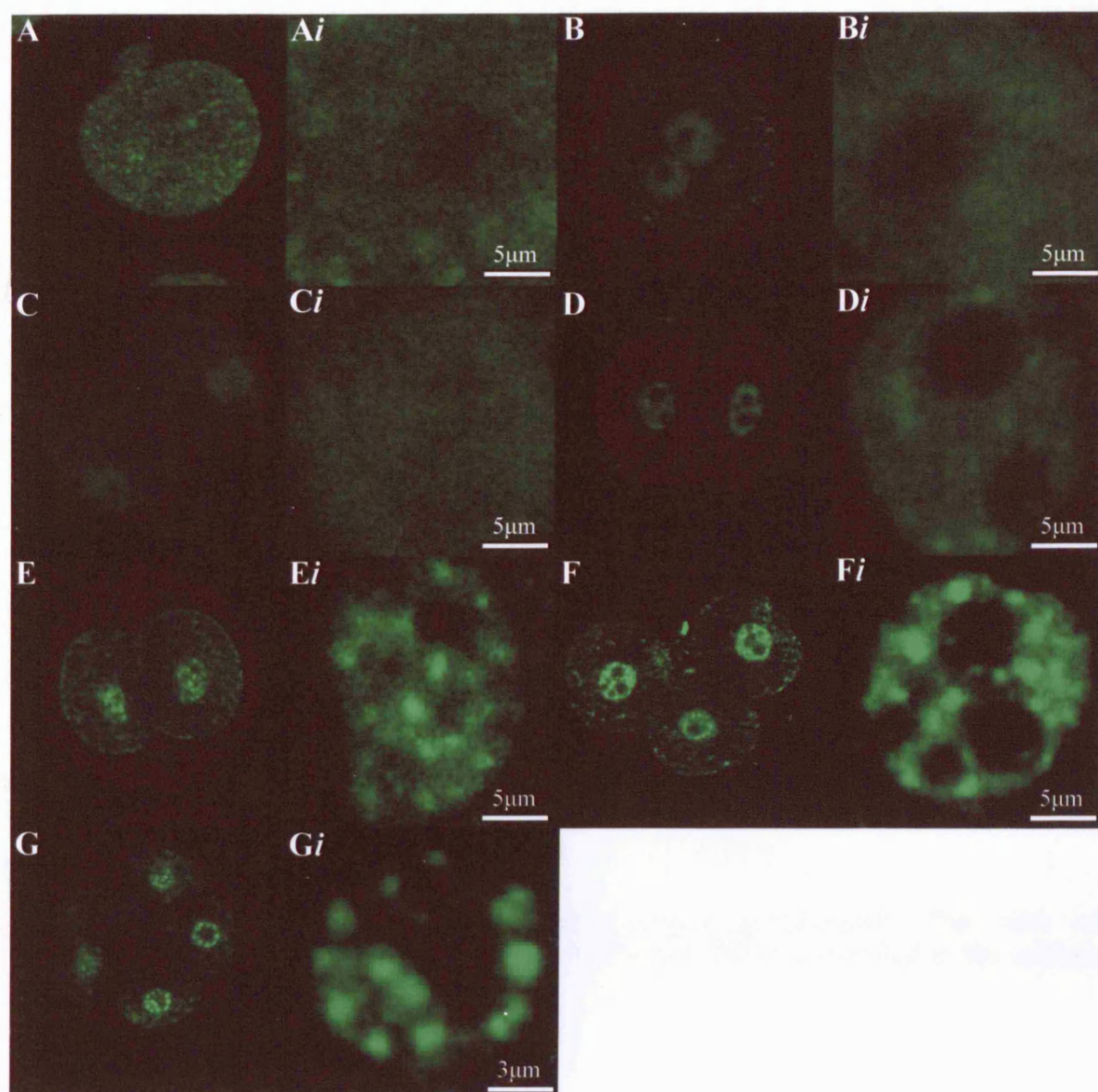


Figure 4.2. The localisation of SC35 during early embryo development. In all images *i* corresponds to magnified image of the nucleus. **A.** In pronucleus stage embryo (26h post hCG), SC35 is distributed uniformly in the cytoplasm. **B.** In late pronucleus stage embryos (30h post hCG), SC35 localises the pronuclei, without any speckles being formed. SC35 localises in the nuclei of the early embryos thereafter, in early 2-cell embryo (36h post hCG) (**C**), mid 2-cell embryo (48h post hCG) (**D**), late 2-cell embryo (60h post hCG) (**E**), 4-cell embryo (**F**) and 8-cell embryo (**G**). Speckles of SC35 are seen after the late 2-cell stage.

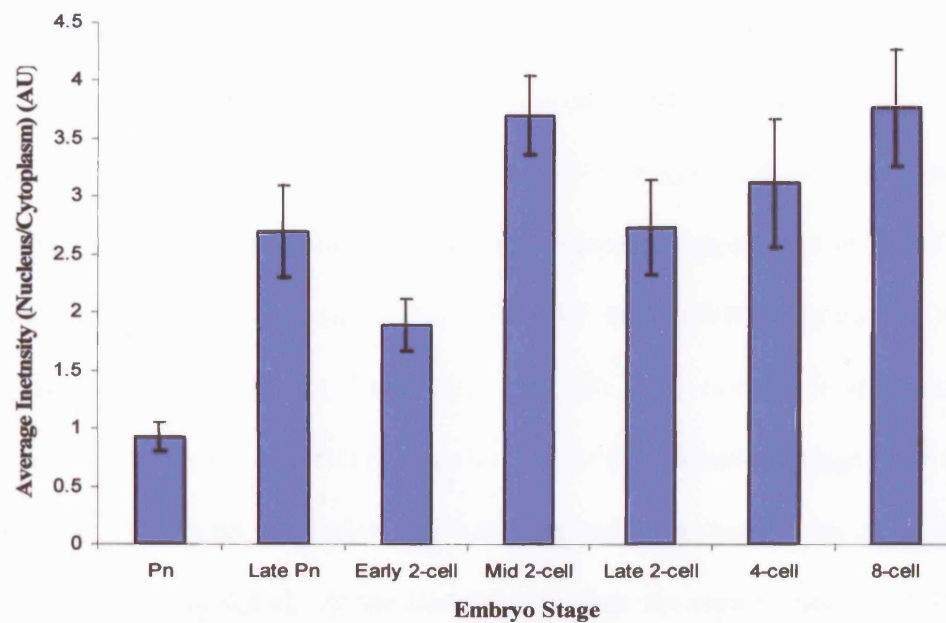


Figure 4.3. SC35 distribution during early embryo development. The ratio of nuclear/cytoplasmic SC35 intensity is shown in the graph. SC35 is recruited to the nucleus after the late pronucleus stage.

4.2.3 PIP₂ localisation during early development

To determine the localisation of nuclear PIP₂ during early development and investigate whether SC35 and PIP₂ co-localise in early mouse embryos, we stained oocytes and embryos using immunofluorescence with the 2C11 antibody, previously characterised by Osborne and colleagues. The localisation of PIP₂ varies throughout development. Cumulus-enclosed GV-stage oocytes show a uniform localisation of PIP₂ within the GV (Figure 4.4 A). In contrast, in cumulus-free GV-stage oocytes there is a speckled appearance in the GV, with speckles of $2.15 \pm 0.5 \mu\text{m}$ (n=6) (Figure 4.4 B). After GVBD, the nuclear speckles disappear (Figure 4.4 C). The staining in MII stage oocytes is identical to the staining shown after GVBD (Figure 4.4 D). At the pronucleus stage there is a low level of PIP₂ in the pronuclei, which is uniformly distributed with no evidence of speckles (Figure 4.4 E). At the late two-cell stage the concentration of PIP₂ in the nucleus is similar to that seen in the pronucleus stage, with no evidence of speckles (Figure 4.4 F). Some PIP₂ immunofluorescence is also observed in the apical plasma membrane. As development of the embryos progresses through the 4- and 8-cell stage, the level of PIP₂ immunoreactivity in the nuclei increases (Figure 4.4 G and H). The appearance of speckles is shown in 4 and 8-cell embryos with speckle size of $1.25 \pm 0.2 \mu\text{m}$ (n=6) and $1.4 \pm 0.25 \mu\text{m}$ (n=6) respectively. In control GV-stage oocytes (Figure 4.4 I), there was no staining in the nucleus or the cytoplasm. In order to determine whether PIP₂ and SC35 co-localise in early development, an attempt was made to perform co localisation

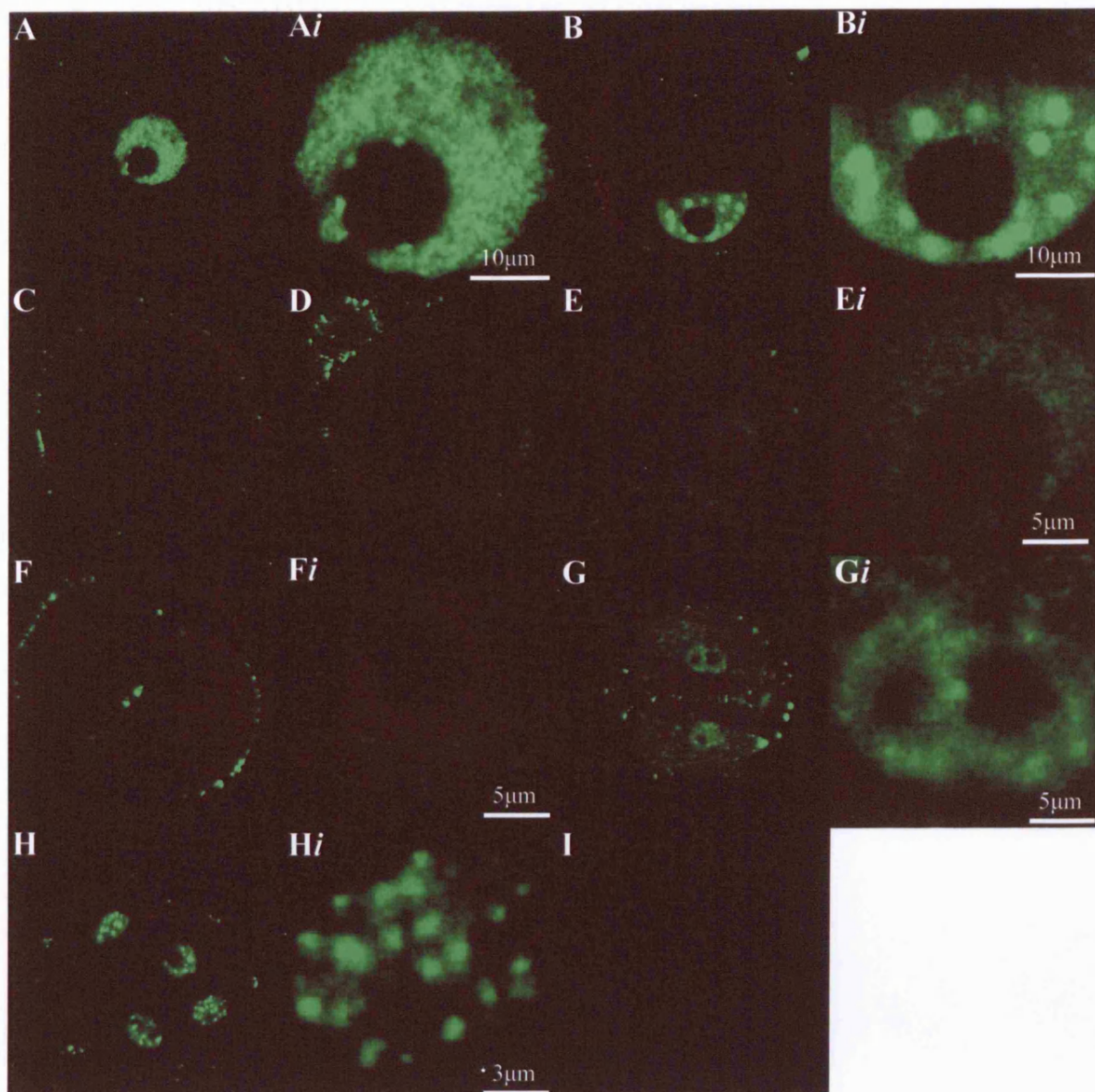


Figure 4.4. The localisation of PIP_2 during early mouse development. In all images *i* corresponds to magnified image of the nucleus. In GV oocytes (A) enclosed in cumulus cells when released from the follicle, PIP_2 is distributed uniformly in the nucleus of the cell. In contrast, GV oocytes, that were free of cumulus cells upon release from the follicle, PIP_2 appears in a speckled pattern within the GV (B). After GVBD (C) and in MII stage oocytes (D) nuclear speckles disappear. From the pronucleus stage (E) onwards PIP_2 localises in the nuclei of the embryo, in late 2-cell embryos (F), 4-cell embryos (G) and 8-cell embryos (H). The first speckles appear at the 4-cell stage in contrast to the SC35 speckles that appear in the late 2-cell stage. I. A control oocyte, stained only with the secondary fluorescent antibody.

experiments using Cy3 labelled 2C11 and the SC35. Unfortunately, the Cy3-labelled 2C11 antibody failed to provide consistent results and no labelling was detectable, despite several attempts.

4.2.4 Speckles are not essential for normal splicing activity

The absence of SC35 and PIP₂ containing nuclear speckles at the one-cell stage provides an experimental model to test whether splicing activity is present in nuclei with no obvious nuclear speckles. To determine whether splicing is taking place in such embryos the TN24 probe was injected into the male pronucleus of early embryos (26h post hCG). Splicing activity was recorded in 1-cell embryos, 8 hours after injection of the probe. The data show that splicing efficiency is comparable to that seen in 2-cell stage embryos (Figure 4.5).

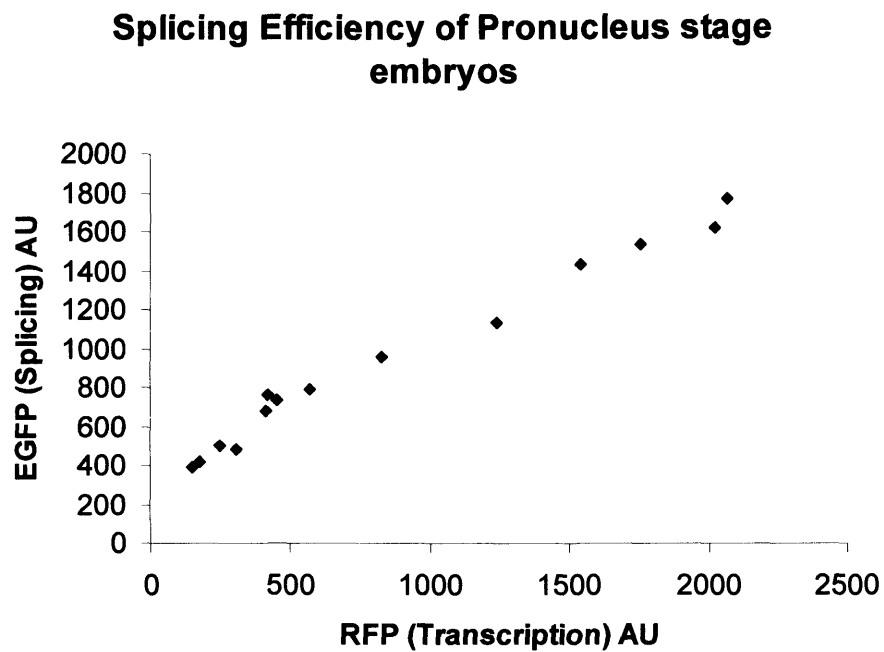


Figure 4.5. Splicing activity of pronucleus stage embryos. Pronucleus stage embryos were injected with the TN24 probe 26 hours post hCG and were cultured for 8 hours. Pronucleus stage embryos are splicing normally despite the fact that there are no apparent speckles seen of either SC35 or PIP₂ in the immunofluorescence data.

4.3 Conclusions

SC35 is a ubiquitous SR protein that plays an important role in splicing by mediating spliceosome assembly and selection of splice sites. In mouse oocytes and embryos we find dramatic differences in the nuclear and cytoplasmic organisation of SC35. In GV-stage oocytes the organisation of SC35 is dependant on the stage of oocyte development, the presence or absence of cumulus cells at the time of oocyte recovery and on the time after release from the follicle.

In growing oocytes the SC35 was distributed in a reticular pattern with numerous small foci of fluorescence. In fully grown cumulus-intact GV-stage oocytes nuclear speckles had increased in size and could be identified as discrete units. In cumulus-free oocytes the speckles were larger than those present in cumulus-intact oocytes. The speckles increased further in oocytes cultured one hour after collection. The change in speckle organisation in maturing oocytes was not affected by the presence of IBMX to inhibit GVBD. This indicates that nuclear reorganisation progresses independently of cell-cycle, at least up to the point at which IBMX inhibits meiotic progression.

This change in the nuclear distribution of SC35 may be explained by the transcriptional state of the oocyte. Growing oocyte are highly transcriptionally active and this stops around the time the oocyte reaches full size (Zuccotti *et al.*, 1995; Bouniol-Baly *et al.*, 1999). This co-insides with the time the speckles are increasing in size. One hour after release from the follicle, when transcription has ceased, the speckles have coalesced into a smaller number of larger foci. The

difference between cumulus-free and cumulus-intact oocyte may also reflect transcriptional state. Cumulus-intact GV-stage oocytes are thought to arise from healthy developing follicles in which normal cumulus-cell interactions are in place. In contrast, cumulus-free oocytes are assumed to arise from antral follicles in which the cumulus-oocyte junctions have broken down. In such oocytes, transcription has probably been inhibited, due to abnormal development.

It would be interesting in future experiments to correlate more directly the morphology of the nuclear speckles with the transcriptional state of the oocyte. In somatic cells, treatment with α -amanitin to inhibit transcription triggers the formation of few large “speckles” (Bregman *et al.*, 1995). It is thought that such speckles act as storage units for the mRNA processing machinery. As transcription is initiated the large aggregates break up to smaller structures ultimately to the size of spliceosomes that are actively involved in mRNA splicing.

Changes in SC35 organisation are also apparent during early development. In MII oocytes, SC35 is distributed throughout the cytosol. After fertilisation, the Pn accumulates SC35, but in the early stages of Pn formation, the ratio of nuclear/cytoplasmic SC35 is close to one. As the cell cycle proceeds, the ratio increases as a result of an increased nuclear accumulation of SC35. The explanation for this increase is not known. It may result from the activation of nuclear transport or as a result of post-translational modification of SC35. In nematodes there is a dephosphorylation of SR proteins during early development

that is associated with the increase in nuclear localisation. The precise mechanism, by which SC35 is regulated by phosphorylation leading to accumulation of SC35 in the late 1-cell pronuclei, is not known. In-vitro studies show that an intermediate level of phosphorylation of SR proteins is required for splicing to take place. Hypo or hyper-phosphorylated states of these proteins cause splicing inhibition (Cao *et al.*, 1997; Prasad *et al.*, 1999). In nematodes, prior to ZGA, it has been shown that SR proteins are in a hyperphosphorylated state and localise to the cytoplasm. After ZGA, partial dephosphorylation activates SR proteins and recruits them to the nucleus (Sanford and Bruzik, 1999; Sanford and Bruzik, 2001). On the other hand disassembly of the nuclear speckles has been shown in studies where overexpression of SR protein specific kinase 2 (SRPK2) increases the phosphorylation state of SR proteins (Kuroyanagi *et al.*, 1998).

The nuclear accumulation of SC35 does not lead to the formation of detectable nuclear speckles at the 1-cell stage. Speckles are evident in 2-cell embryos at the stage we know splicing has been initiated. The absence of nuclear speckles at the one-cell stage is surprising since experiments suggest that splicing and mRNA processing are initiated at this stage of development (Hamatani *et al.*, 2004a; Zeng and Schultz, 2005). We have tested this directly by injecting the splicing reporter into 1-cell embryos. The 1-cell embryo is clearly highly capable of splicing mRNA, despite the apparent lack of nuclear speckles. The relationship of speckles between the 1-cell and 2-cell stage is not understood. It is unlikely that

PIP₂ forms a nuclear scaffold for co-ordinating this process as it coalesces into speckles even later than SC35.

The fact that PIP₂ does not form speckles at the 2-cell stage like SC35, but forms speckles only later in development, suggests that it may not be involved in the regulation of splicing as suggested by Osbourne and colleagues. Depletion of PIP₂ in HeLa extracts inhibited splicing activity by approximately 80%. However, such depletion probably involved the depletion of PIP₂ associated proteins (possibly SC35), therefore yielding inhibition of splicing. These findings reported in the present study suggest that PIP₂ is not involved in regulation of splicing or SC35 mediated spliceosome assembly at the 2-cell stage. More evidence of precise timing of PIP₂ and SC35 co-localisation could have been achieved with the Cy3 labeled PIP₂ antibody. Unfortunately the Cy3 labeled PIP₂ antibody did not yield any reliable data.

The experiments reported here describe the developmental changes in nuclear structures associated with splicing factors such as SC35. The embryo will provide a useful model system in which to study the mechanism of spliceosome assembly and disassembly.

5. The role of SRp38 in early mouse development

5.1 Introduction

SRp38 is a recently identified protein that belongs to the SR (Serine/Arginine rich) protein family. SRp38 appears in a variety of studies with a different name. Therefore SRp38 is also known as fusip1 (Komatsu *et al.*, 1999), NSSR-1 (Komatsu *et al.*, 1999), TASR-2 (Clinton *et al.*, 2002) and SRrp40 (Cowper *et al.*, 2001). The structure of SRp38 is typical of an SR protein, with an N-terminal RNP-type RNA binding domain and a C-terminal region rich in serine-arginine dipeptide repeats (RS domain) (Shin and Manley, 2002). SRp38 is widely expressed. mRNA has been identified in the brain, testis (Komatsu *et al.*, 1999), ovary, thymus, spleen, heart skeletal muscle and placenta (Cowper *et al.*, 2001). In HeLa cells SRp38 localises in the nuclear speckles but shuttles to the cytoplasm when transcription in the cell is inhibited (Cowper *et al.*, 2001).

The functions of SRp38 are still under investigation and have not yet been fully elucidated. SRp38 was first described as a regulator of splicing, when it was found to enhance splicing activity in vitro (Komatsu *et al.*, 1999). Such a role has

proved controversial and further studies have not confirmed a role in enhancing splicing. SRp38 is also not sufficient to restore splicing activity in cytoplasmic S100 extracts from HeLa cells. Subsequent studies showed in S100 extracts that SRp38 had a dominant negative effect on SC35 and SF2/ASF dependant splicing in vitro (Cowper *et al.*, 2001;Shin and Manley, 2002). This suggested a role of SRp38 in splicing repression, an unexpected property for an SR protein, since most SR family members can activate splicing in vitro.

It was later shown that SRp38 is a potent repressor of splicing and that the inhibition of splicing by SRp38 is regulated by phosphorylation. In two conditions, in which splicing is inhibited, mitosis and heat shock, SRp38 is activated by dephosphorylation (Shin and Manley, 2002;Shin *et al.*, 2004). The phosphatase responsible for dephosphorylation of SRp38 is not yet known. Evidence for a role in inhibiting splicing is shown by the activation of splicing after depletion of dephosphorylated SRp38 (dSRp38) and the inhibition of splicing after addition of dSRp38 to the same extracts. The mechanism of dSRp38 dependant inhibition appears to be due to its ability to interact with U1 snRNP (Shin *et al.*, 2004) and in particular U1-70k (Fushimi *et al.*, 2005) and interfere with splice site recognition, by preventing the formation of complex A, the initial step in splicing. Increased amounts of SC35 could not restore splicing activity suggesting that other SR proteins are not the target of splicing inhibition (Shin *et al.*, 2004).

An alternative mechanism has been suggested by Cowper and colleagues. They propose that SRp38 antagonises the activity of SR proteins such as SC35 and SF2/ASF and inhibits splicing by exerting a dominant negative effect on their function. Furthermore, SRp38 activated splicing of the most distal alternative 5' splice sites of adenovirus E1A pre-mRNA in in vitro assays. This activity was shown to depend on the N-terminal RNA binding domain of SRp38 (Cowper *et al.*, 2001), suggesting a further role of SRp38 in alternative splicing. Such a role was also shown by expression of SRp38 in NIH3T3 cells, where a decrease in a particular exon exclusion was demonstrated compared to control cells (Komatsu *et al.*, 1999).

Recent studies have demonstrated a potential novel role of SRp38. SRp38 was shown to regulate neurogenesis in *Xenopus* embryos (Liu and Harland, 2005). Overexpression of SRp38 blocked the activity of neurogenin, a protein essential for normal neurogenesis, leading to inhibition of neuronal differentiation. In the same study, depletion of SRp38 increased neurogenesis by activation of a Delta ligand, responsible for differentiation of cells into neurons in early development. There was no evidence that this regulation of neuronal differentiation was due to splicing inhibition or aberrant splicing of a particular gene, such as neurogenin. The mechanism of inhibition is thought to be mediated by an SRp38 interaction with the 28S rRNA. This might have an effect in the regulation of ribosome biosynthesis or function (Liu and Harland, 2005).

In this chapter I have investigated the role of the SRp38 during early mouse development. Initial experiments present the localisation of the protein during early mouse development. Further experiments demonstrate that overexpression or depletion of the protein caused the arrest of the oocyte or the embryo, without any affect in the splicing efficiency of the cell when assayed with the TN24 probe.

5.2 Results

5.2.1 Characterisation of the SRp38 antibody.

We firstly wanted to identify whether SRp38 is present in mouse embryos and whether we could see the same pattern of cell-cycle dependent dephosphorylation in mouse embryos. The SRp38 antibody was developed by Cocalco biological and was provided by James Manley as a gift. Control embryos were collected at the 52h post hCG (mid 2-cell stage). To obtain embryos arrested in mitosis, nocodazole was included in the culture medium to induce an M-phase arrest. Culture of embryos in nocodazole for 12 hours resulted in a population of mitotic 2-cell embryos as evidenced by the absence of nuclei. Western blotting was performed on proteins from control and mitotic 2-cell embryos. The blots revealed two bands of the same molecular weights in the samples. One band appears to be of size ~35kDa and correlates with the full length SRp38 which is 262 amino acids (Shin and Manley, 2002). Previous data reports that SRp38 in mouse brain, testis and spinal cord runs in the region of 35kDa rather than 38kDa (Komatsu *et al.*, 1999; Liu *et al.*, 2003). The second band ran at ~21kDa and correlates with the truncated form of SRp38, which is 183 amino acids (Komatsu *et al.*, 1999). However, there is no apparent band in the region of ~28kDa where the dephosphorylated form of SRp38 has been previously reported to run (Shin and Manley, 2004). The phosphorylation state of SRp38 could also be confirmed by treatment of control cells with phosphatase and running them on the same Western blot. It can therefore be argued that the first band represents the phosphorylated form of SRp38. This suggests that in mouse embryos, SRp38 is in

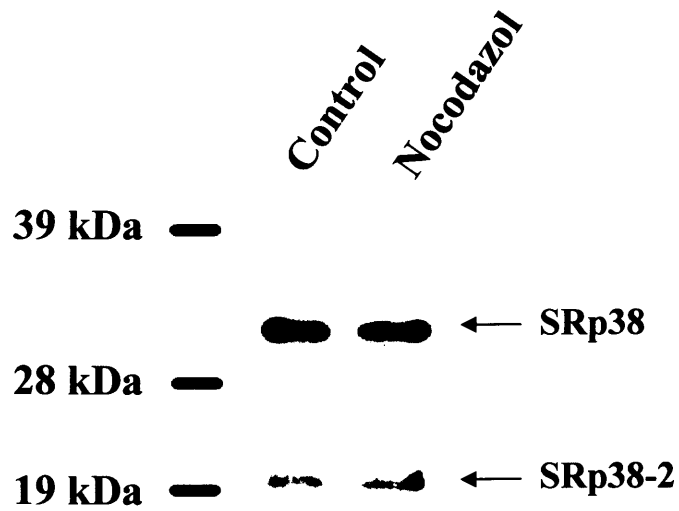


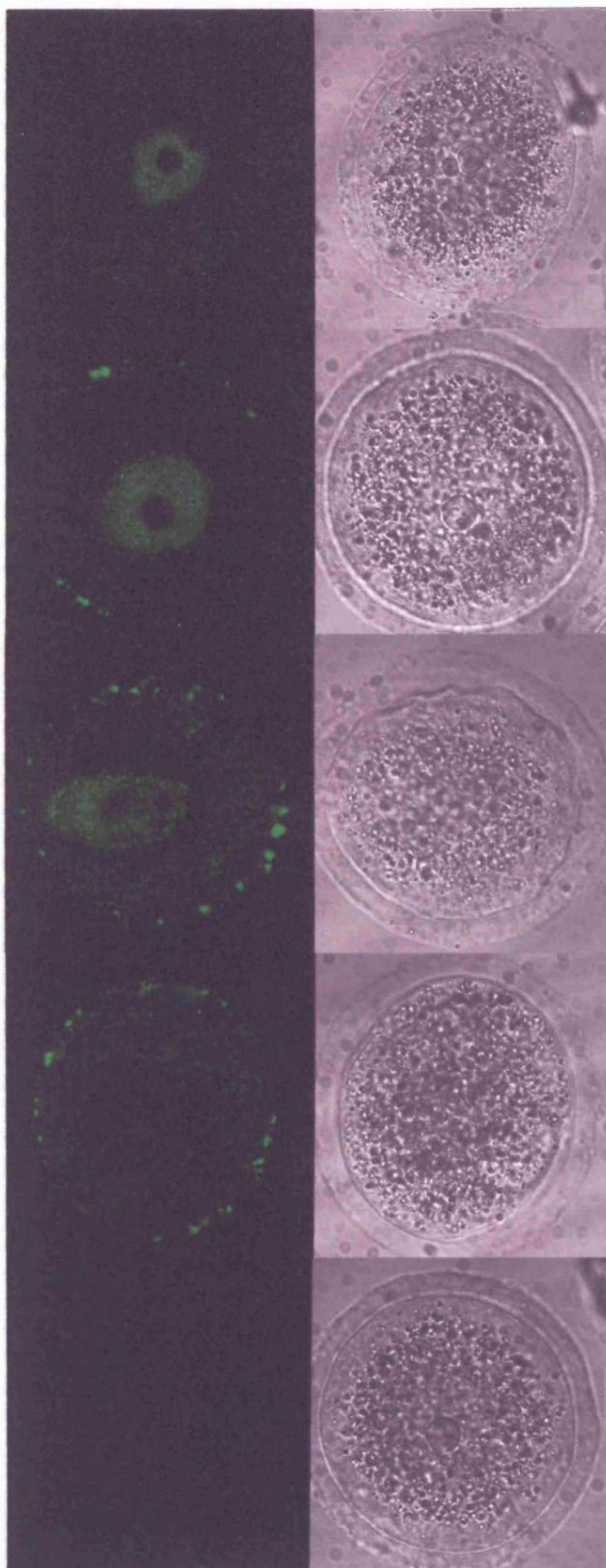
Figure 5.1 SRp38 runs at the same molecular weight in interphase and mitosis (nocodazol) in mouse embryos. The band ~35kDa represents the full length SRp38 and the ~21kDa represents the truncated form of SRp38 known as SRp38-2. The fact that the protein levels between controls and nocodazol treated embryos are the same show that there is no cell-cycle dependant change in the phosphorylation state of SRp38.

its phosphorylated state. The lack of any shift in molecular weight in the nocodazole arrested mitotic embryos suggests that there is no cell cycle-dependant change in the phosphorylation of SRp38 in mouse embryos (Figure 5.1).

5.2.2 SRp38 localisation during early development using immunofluorescence

Following the confirmation that SRp38 is expressed in mouse embryos, the next step was to investigate its localisation using immunofluorescence. SRp38 was found to localise mainly in the nucleus of the oocyte or embryo. Figure 5.2A shows the localisation of SRp38 during oocyte maturation. In the GV-stage the protein was uniformly distributed in the nucleus in the pattern of small speckles. After GVBD the protein was distributed uniformly throughout the cytoplasm, with an accumulation of immunoreactivity on the spindle during MI. A similar cytoplasmic immunofluorescence was seen at MII, although no immunoreactivity was evident on the spindle. In control GV-stage oocytes, there was no staining seen in the nucleus or cytoplasm. Following fertilisation, SRp38 was recruited to the two pronuclei of the embryo with a pattern similar to that seen in GV-stage oocytes (Figure 5.2B). After NEBD, the protein was distributed throughout the cytoplasm with an accumulation of the protein on the developing spindle. From the two-cell stage onwards the protein localised in the nucleus, but the appearance of its distribution was uniform with speckles appearing as the embryo developed to the late 2-cell stage (Figure 5.2B).

5.2 A



GV

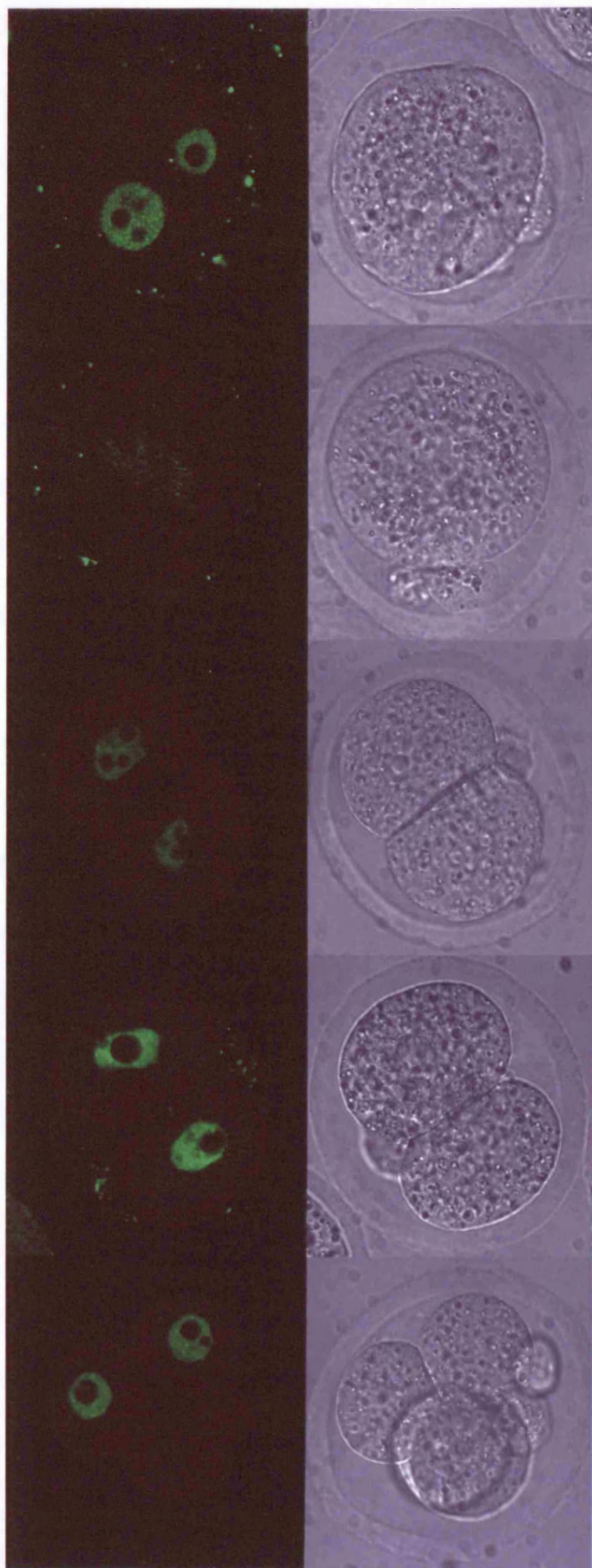
GV + 1h

GV + 3h

MI

Control

5.2 B



Pronucleus

NEBD

Mid 2-cell
(48h post hCG)

Late 2-cell
(60h post hCG)

4-cell

5.2.3 The distribution and effect of SRp38-EGFP in one and 2-cell embryos

The immunofluorescence experiments and Western blot experiments showed that SRp38 is expressed in early mouse oocytes and embryos. To monitor the localisation of SRp38 in live cells and to determine the effect of overexpression in early development, an SRp38-EGFP vector was constructed by cloning the SRp38 gene into pEGFP-N1 (Clontech). mRNA was prepared using the vector as a template, PCR amplification of SRp38-EGFP and in vitro transcription and polyadenylation (as described previously).

To determine the localisation of SRp38 in 1-cell and 2-cell embryos, mRNA was microinjected to pronucleus-stage embryos (26h post hCG). The fusion protein was expressed and found to be localised in the pronuclei within 3 hours of microinjection. (Figure 5.3 i, ii). After NEBD, SRp38 was uniformly distributed in the cytoplasm of the embryo (iii, iv). In mid 2-cell embryos (~48 hours post hCG), SRp38 localised in the nuclei of the embryo (v, vi). The pattern of localisation was the same as that demonstrated earlier with immunofluorescence. The only difference in localisation is seen after NEBD in the immunofluorescence experiments where there is some SRp38 localising in the spindle. This data confirms the nuclear localisation but not the spindle localisation obtained by immunofluorescence. Furthermore the data shows that the EGFP does not inhibit the localisation of SRp38 and suggests that SRp38-EGFP has similar properties as SRp38.

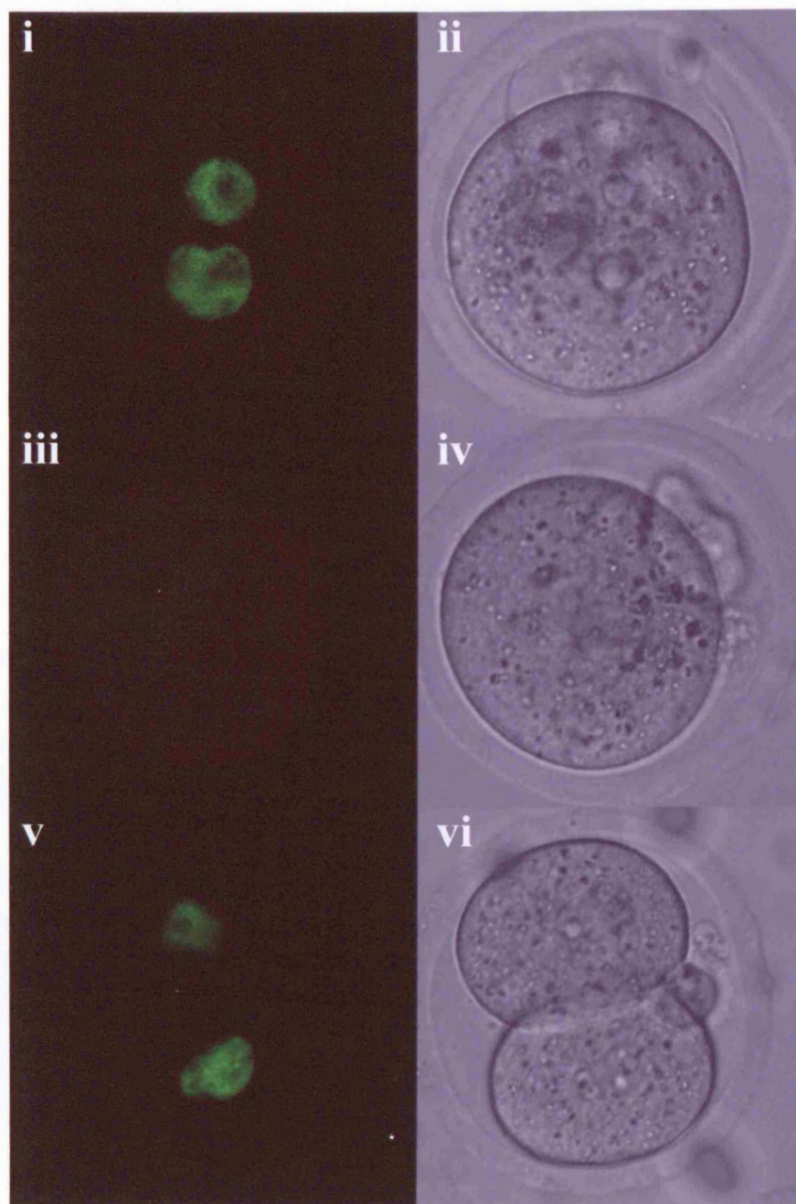


Figure 5.3. Expression of SRp38-EGFP after mRNA injection at the pronucleus stage. SRp38 localises in the pronuclei (i, ii). After NEBD (iii, iv) the protein is uniformly distributed in the cytoplasm of the 1-cell embryo. After cleavage to the 2-cell stage, the SRp38-EGFP once again localised to the nuclei (v, vi).

| | n | % of embryos developing after SRp38 over-expression | | | |
|----------------|----|---|------|--------|--------|
| | | Pronucleus | NEBD | 2-cell | 4-cell |
| Control | 52 | 0 | 0 | 6 | 94 |
| SRp38-Injected | 46 | 0 | 0 | 100 | 0 |

Table 5.1. Over-expression of SRp38 after mRNA injection at the pronucleus stage inhibits early development. SRp38 overexpressing oocytes arrest at the 2-cell stage. Control EGFP mRNA injected embryos progress normally to the 4-cell stage.

Overexpression studies are also important to determine the effects of the protein in normal development. All 1-cell embryos expressing the SRp38-EGFP progressed normally to the 2-cell stage, but they did not progress any further. This inhibition was not due to the EGFP since the majority of control embryos injected with mRNA encoding EGFP progressed normally to the 4-cell stage. This result suggests that SRp38 inhibits development beyond the 2-cell stage, but further experiments are necessary to determine whether the effect is not due to different concentrations of SRp38 at 1 and 2-cell stages (see below) (Table 5.1).

5.2.4 The effects of SRp38 on development and splicing efficiency

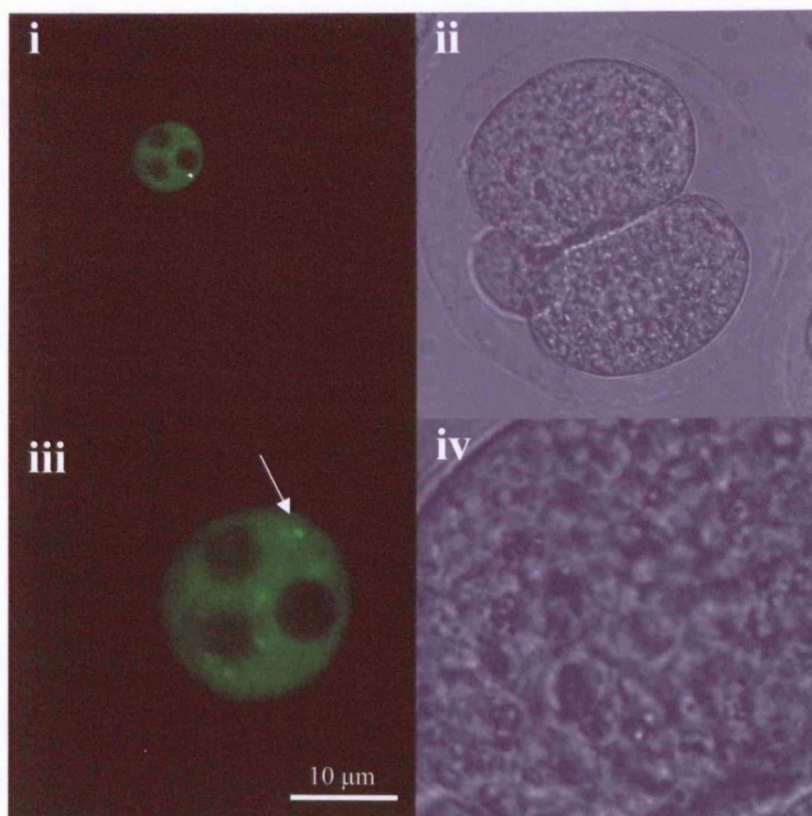
To examine the effects of SRp38 on 2-cell stage development and splicing efficiency, SRp38-EGFP plasmid DNA was injected into the nuclei of single blastomeres of 2-cell embryos, 42 hours post hCG. The embryos were monitored at ~58 hours (Figure 5.4 A), and showed that the SRp38-EGFP localised in the nucleus of the expressing blastomere with the appearance of some speckles seen at higher magnification (Figure 5.4 A iii, iv).

To determine whether this modified injection regime had a similar effect on development, the SRp38-EGFP-injected embryos were cultured overnight for 16-18 hours. Following culture, the injected blastomere with SRp38-EGFP was arrested, whereas the sister blastomere had divided normally, resulting in a 3-cell embryo (Figure 5.4 B). To ensure that the EGFP had no effect on the development of embryos, pEGFP-N1 was injected into the nucleus of a single blastomere of a

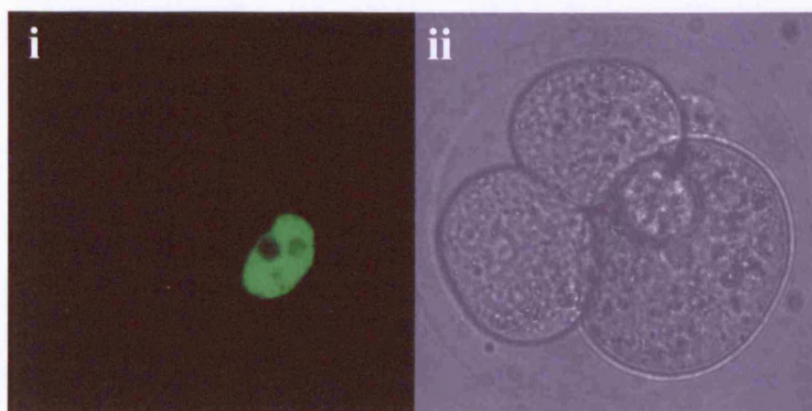
2-cell embryo. As seen in Figure 5.4C the 2-cell embryo has developed normally to the 8-cell stage, with the EGFP apparent both in the cytoplasm and the nuclei of the embryos. All SRp38-EGFP injected blastomeres failed to cleave (n=24), as previously reported, whereas ~80% of blastomeres expressing EGFP alone, progressed normally to the 4-cell stage (n=14) (Figure 5.4D).

We then tested whether the ability of SRp38-EGFP to inhibit development was correlated with an inhibition of splicing. This experiment was also useful to assess whether SRp38-EGFP interfered with the onset of EGA. The same procedure described above was performed to over-express SRp38 in 2-cell embryos. To examine the effect of SRp38 overexpression on splicing, the TN24 probe was microinjected into a blastomere of a 2-cell embryo overexpressing SRp38. The embryos were cultured and monitored for fluorescence 8 hours after injection of the splicing reporter. To ensure that the green fluorescence of the SRp38-EGFP did not interfere with the EGFP expressed from the splicing probe, fluorescence was collected from a region of interest that excluded the nucleus. This avoided contamination of the probe signal by the nuclear localised SRp38 and allowed the estimation of splicing activity as described in the previous chapter. In both Figures 5.2A and 5.3A, it is clear that SRp38 localised in the nucleus of the early embryos, with no detectable fluorescence in the cytoplasm. Furthermore, if there was some EGFP in the cytoplasm due to SRp38-EGFP, the low red fluorescence values of the probes

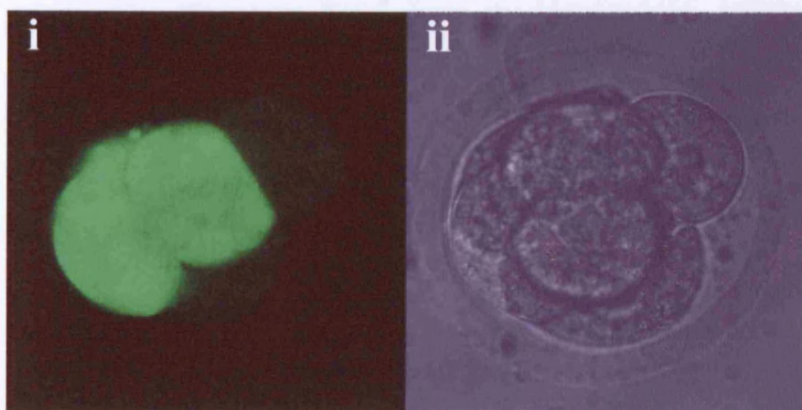
5.4 A



B



C



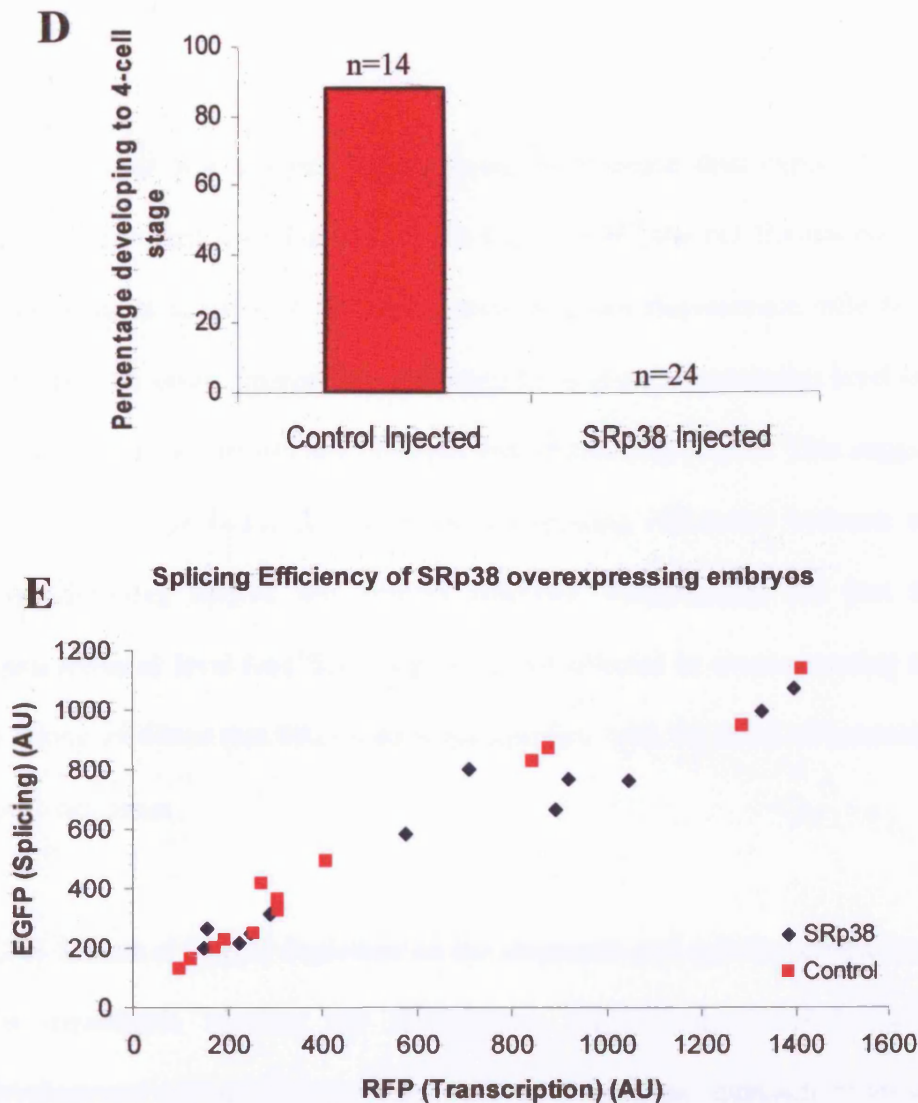
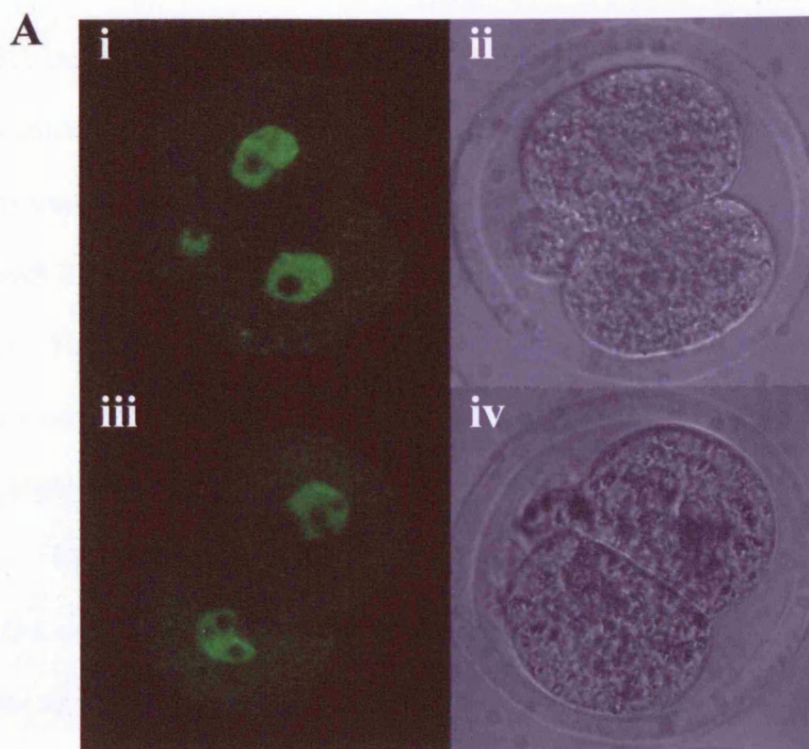


Figure 5.4. Overexpression of SRp38 in late 2-cell embryos and effect of overexpression on splicing efficiency. **A.** SRp38-EGFP localises in the nucleus of the embryo (i, ii). Some aggregates of fluorescence (speckles) can be seen at higher magnification (iii, iv) (shown by arrowhead). **B.** SRp38-EGFP injection into 2-cell embryos causes developmental arrest in the injected blastomere (i, ii). Bright field image shows a 3-cell embryo and fluorescence image shows SRp38-EGFP localised to the nucleus of the injected blastomere. The control blastomere has divided normally. **C.** Injection of pEGFP-N1 has no effect on development of injected blastomeres (80% cleave). This confirms that injection technique and EGFP do not cause cleavage arrest and developmental effects are a result of SRp38.. Overexpression of SRp38 in late 2-cell embryos and effect of overexpression on splicing efficiency. **D.** Data showing rates of development to the 4-cell stage (n=24). Note that none of the SRp38-EGFP-injected blastomeres cleave to allow development to the 4-cell stage, while 80% of the control blastomeres cleave. **E.** Overexpression of SRp38 does not affect splicing efficiency. Overexpressing SRp38 embryos were injected with the TN24 probe at the mid 2-cell stage. Each point on the graph represents a blastomere of a 2-cell embryo at the late 2-cell stage.

would appear with a much higher green fluorescence than expected. However, such a result was not obtained. In the Figure 5.4E, the red fluorescence reflects transcriptional activity of the probe whereas green fluorescence reflects splicing activity. The green fluorescence intensity for a given transcription level is shown to be similar in controls and embryos overexpressing SRp38. This suggests that there is no significant difference in the splicing efficiency between embryos overexpressing SRp38 and control embryos. Furthermore, the fact that the transcriptional level (red fluorescence) is not affected in overexpressing embryos is strong evidence that SRp38 does not interfere with the onset of transcription at the 2-cell stage.

5.2.5 Effect of SRp38 depletion on development and splicing efficiency

To investigate whether the endogenous SRp38 protein is important for development and splicing we have used a morpholino approach in an effort to deplete the SRp38 protein from 2-cell embryos. To allow a maximum opportunity for endogenous SRp38 to turnover, the morpholinos were injected at the one cell stage. To confirm that the morpholino injection resulted in depletion of the SRp38 protein, embryos were microinjected with an SRp38 morpholino or a control scrambled morpholino. The levels of SRp38 were examined using immunofluorescence and Metamorph analysis (Figure 5.5A). Immunofluorescence revealed that SRp38 was reduced to ~30% of that seen in control embryos when nucleus/cytoplasm pixel intensities were compared (Figure



B Effect of Morpholino on SRp38 levels in 2-cell embryos

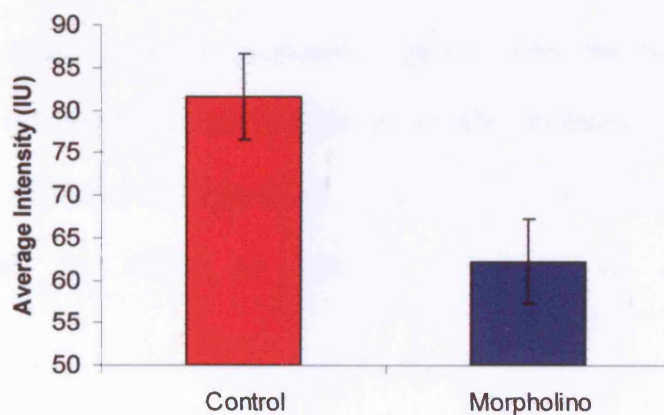
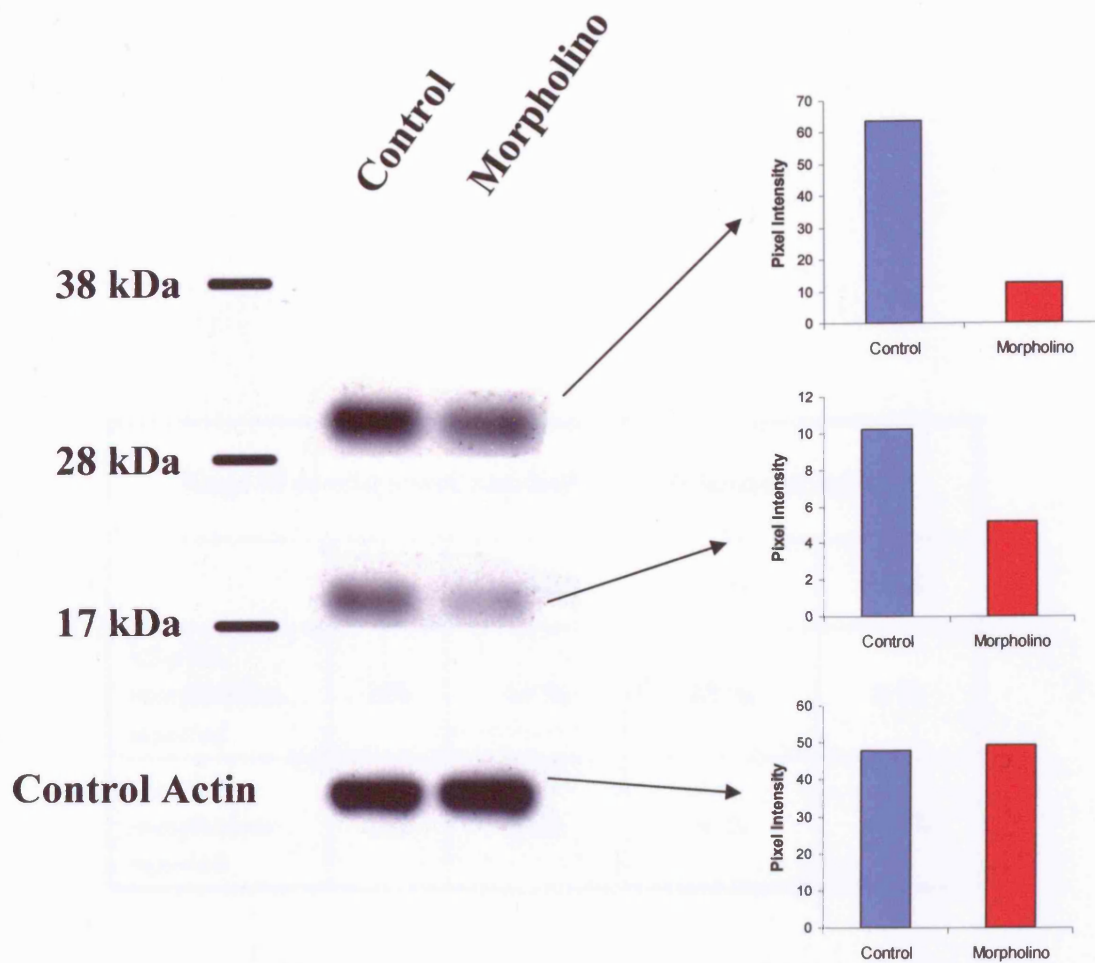
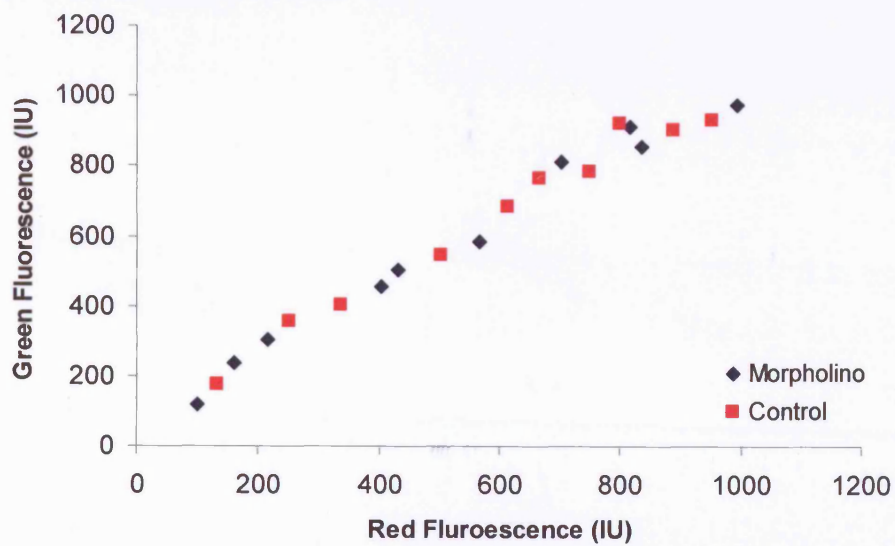


Figure 5.5. SRp38 morpholino decreases the level of SRp38 immunoreactivity. A i to A iv show immunofluorescence and bright field images of control (i, ii) and morpholino- injected (iii, iv) 2-cell embryos. **B.** Graph shows the difference in fluorescence between control and morpholino injected embryos.

5.5B). A western blot of control and morpholino-injected embryos showed a reduction in the level of SRp38 protein in the morpholino-injected embryos. The blots were re-probed for actin to confirm that similar levels of protein were loaded in each lane. The graphs beside each band show the mean pixel intensity of each band. The level of SRp38, as determined by pixel intensity, decreased by 70%. The level of the truncated SRp38 was reduced by 50%. Actin was not affected by the treatment with the morpholino (Figure 5.6).

The effect of SRp38 depletion in development was monitored by culturing control and SRp38 morpholino injected embryos to the 4-cell stage. Ten percent of the morpholino injected embryos arrested at the 1-cell stage with the remaining embryos subsequently arresting at the 2-cell stage. Control embryos (95%) developed to the 4-cell stage when the culture was terminated (Table 5.2).

In order to determine whether the splicing efficiency of the embryos was affected following depletion of the SRp38, the TN24 probe was injected at the mid 2-cell stage embryos previously injected with the SRp38 or control morpholino (at pronucleus stage). Eight hours after injection of the TN24 probe, the splicing efficiency was examined. The data clearly show that the depletion of SRp38 had no effect on splicing efficiency of 2-cell embryos.

A**B****Effect of SRp38 depletion on splicing efficiency**

| Stage of development reached after 48 hours of culture | | | | |
|--|-----|------------|--------|--------|
| | n | Pronucleus | 2-cell | 4-cell |
| SRp38-morpholino-injected | 108 | 11 % | 89 % | 0 % |
| Control-morpholino-injected | 102 | 0 % | 6 % | 94 % |

Table 5.2. Effect of SRp38 morpholino on embryo development. The majority of control injected embryos develop normally to the 4-cell stage. However, the injection of the SRp38 morpholino has arrested development partially by the pronucleus stage and completely by the 2-cell stage.

5.2.6 Overexpression or depletion of SRp38 also inhibits oocyte maturation

Previous results in 5.2.4 showed that there was no direct or significant effect of the overexpression of SRp38 on splicing efficiency. If the effect of the protein is independent of splicing it may also be expected to have an effect on oocyte maturation, since it is thought to be independent of new transcription. GV-stage oocytes were arrested with IBMX and injected with SRp38-EGFP mRNA. After overnight culture, the GV-stage oocytes were then released from IBMX and cultured for 15 hours. Figure 5.7 shows a GV-stage oocyte, injected with SRp38-EGFP. SRp38-EGFP localised to the GV, with some evidence of speckles around the nucleolus. After release of oocytes from IBMX, SRp38-EGFP-injected oocytes failed to undergo GVBD. Control oocytes and oocyte cultured in α -amanitin (RNA polymerase II inhibitor) underwent GVBD with 100% and 93%, respectively, progressing to MII (Table 5.3).

Depletion of SRp38 from oocytes was also examined to determine whether oocyte maturation would be affected. The GV-stage oocytes were injected with the morpholino and cultured overnight in the presence of IBMX. As for 2-cell embryos, we confirmed that the SRp38 morpholino-injected embryos showed a significant decrease in protein levels to ~30% of that seen in controls injected with a scrambled morpholino (Figure 5.8A, B). The majority of control GV-stage oocytes matured to MII stage at a rate of ~90%. However, ~30% of morpholino

injected GV-stage oocytes arrested at the GV stage upon release from IBMX. The remaining morpholino injected oocytes arrested at the MI stage (Table 5.4).

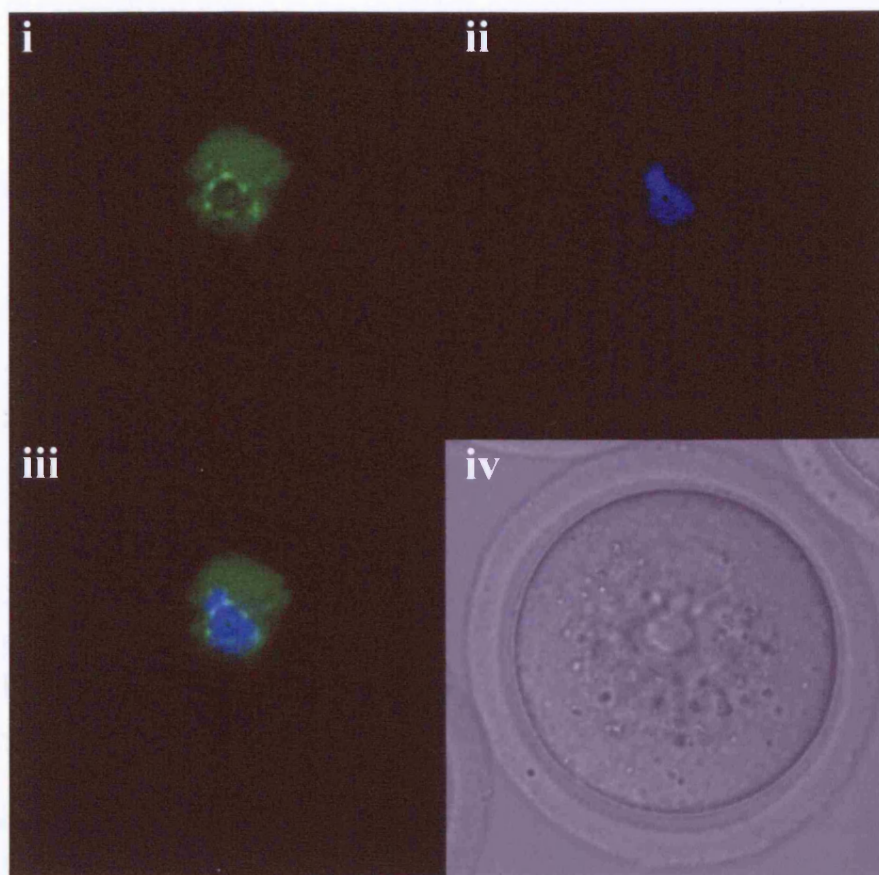
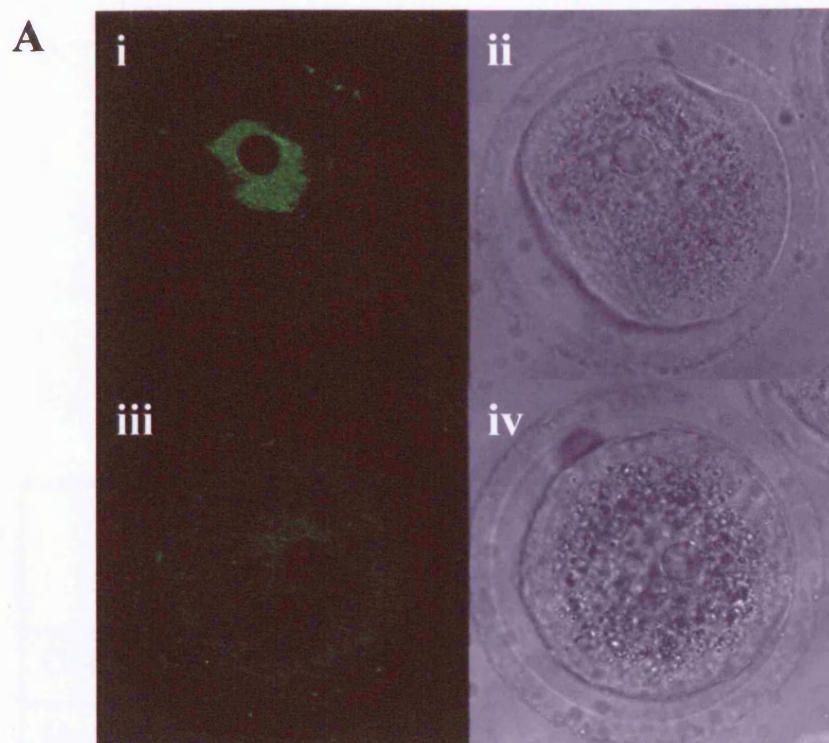


Figure 5.7. Expression of SRp38-EGFP in GV oocytes. The GV oocytes were injected with SRp38 mRNA and cultured overnight in IBMX enriched media. The GV-stage oocytes were released and cultured for 15 hours (i, SRp38-EGFP, ii, Hoechst staining, iii merge image of i and ii, iv, bright field image).

| Stage | n | % of oocytes | | |
|--------------------|-----|--------------|----|-----|
| | | GV | MI | MII |
| Control | 105 | 0 | 0 | 100 |
| α -amanitin | 98 | 0 | 7 | 93 |
| SRp38-EGFP | 78 | 100 | 0 | 0 |

Table 5.3. Effect of overexpression of SRp38 on oocyte maturation. Arrested GV-stage oocytes were injected with SRp38 mRNA and cultured overnight in presence of IBMX. Upon release, all SRp38-EGFP-injected GV oocytes remain arrested at the GV stage. Control oocytes and oocytes cultured in α -amanitin enriched media progress to the MII stage upon release.



B Effect of Morpholino on SRp38 level in GV oocytes

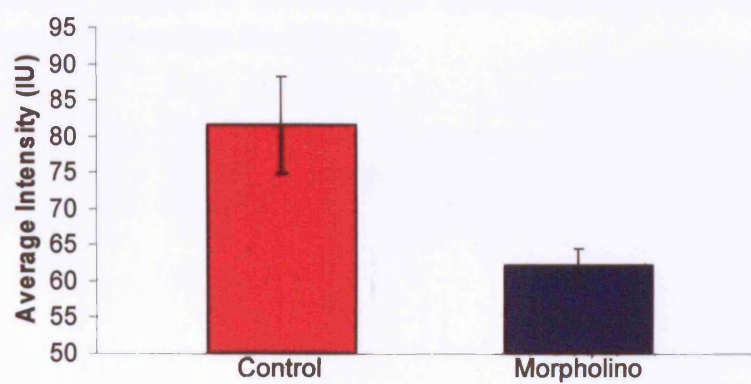


Figure 5.8. Effect of SRp38 morpholino on oocyte maturation. Ai to Aiv show immunofluorescence images of control (i, ii) and morpholino injected (iii, iv) GV oocytes. **B.** Graph shows the reduction in fluorescence between control and morpholino injected GV oocytes.

| | n | % of oocytes | | |
|------------|----|--------------|----|-----|
| | | GV | MI | MII |
| Control | 94 | 0 | 12 | 88 |
| Morpholino | 90 | 27 | 73 | 0 |

Table 5.4. Effect of morpholino on oocyte maturation. The majority of control injected oocytes progress to the MII stage. However, morpholino injected oocytes do not progress further than the MI stage

5.3 Conclusions

This chapter presents the role of the SR protein SRp38 in early mouse development. Western blot results show expression of both full length SRp38 (~35kDa) and the truncated form of SRp38 (~20kDa) in early mouse embryos. In studies on mouse brain, spinal cord and testis tissues, SRp38 has been shown to run at a molecular weight of ~35kDa (Liu *et al.*, 2003). This in contrast to HeLa cell studies in which the protein has been shown to run at 38kDa (Shin and Manley, 2002). The dephosphorylated form of SRp38 has been reported by Shin and Manley to appear in the region of 28kDa. The fact that we failed to detect a band in the region of ~28kDa as well as the fact that SRp38 runs at the same molecular weight in interphase and mitosis (nocodazol treatment), shows that there is no dephosphorylated SRp38 in early mouse embryos. It is unclear why mouse embryos do not show any dSRp38, while evidence does exist in HeLa cells. It may be that the levels of dSRp38 are below the level of detection, or that the dSRp38 phosphatase is not expressed in the oocyte. These results suggest that the dephosphorylation of SRp38 may not be important in regulation of splicing during early mammalian development.

The protein was shown to localise by immunofluorescence or expression of SRp38-EGFP to the nuclei at a number of different stages; GV-stage oocyte, 1-cell embryo and 2-cell embryo. During mitosis the protein distributed to the cytoplasm. Within the nucleus the protein appears to concentrate in nuclear speckles. This localisation of SRp38 in speckles was previously shown in cos7

cells by Cowper and colleagues (Cowper *et al.*, 2001). It was suggested by Cowper and colleagues that the nuclear speckles resemble the localisation of SC35 speckles. No evidence was provided to show co-localisation of the SC35 and SRp38. In the experiments presented here, speckles using the SRp38 antibody and the SRp38-EGFP, appear fewer in number than the speckles detected using SC35 (see previous chapter). To elucidate whether SRp38 and SC35 are co-localised further experiments using fluorescent fusion proteins or dual immunofluorescence labelling experiments are required.

The majority of SR family proteins are known to be splicing activators or enhancers (Graveley, 2000). However, it has been previously demonstrated that SRp38 possesses properties of a splicing repressor, rather than activator or enhancer, especially during mitosis and in response to heat shock (Shin and Manley, 2002; Shin *et al.*, 2004). Our findings are consistent with a role for SRp38 on early development being independent of splicing. Depletion or overexpression of SRp38 inhibited development at the 2-cell stage. These effects were initially shown to be independent of splicing by the use of the TN24 splicing reporter. The TN24 probe was selected due to the fact that it presented a greater splicing efficiency compared to the other introns. In both cases, neither a decrease nor an increase in the splicing efficiency was observed when the TN24 probe was used. This finding may be explained in part by the finding that embryos do not apparently contain any dephosphorylated SRp38, which has been shown to be the most potent form of SRp38 for inhibiting splicing activity (Shin and Manley,

2002). An alternative explanation for not detecting any change in splicing activity may be that the TN24 splicing probe is not targeted or not sensitive enough to detect effects of SRp38 on splicing. For example TN24 will not report alternative splicing activity and previous studies have suggested that SRp38 is involved in splice site selection (Komatsu *et al.*, 1999; Cowper *et al.*, 2001; Nasim *et al.*, 2002). SRp38 has been shown to exert dominant negative effects on the activity of other splicing activators or enhancers (Cowper *et al.*, 2001; Shin and Manley, 2002). As such, the overexpression of SRp38 may interfere with the balance of activity of these splicing activators or enhancers, leading to aberrant alternative splicing. This may be one possible mechanism of action responsible for the developmental arrest caused by modulating levels of SRp38.

The depletion or overexpression experiments performed on GV-stage oocytes argue that the developmental effects are independent of splicing and alternative splicing. GV-stage oocytes do not transcribe and therefore are not actively splicing mRNA. This is illustrated by the experiment with α -amanitin, an RNA Polymerase II inhibitor, where the GV-stage oocytes matured normally to the MII stage. It would be interesting at this stage to find out whether splicing efficiency was affected in GV-stage oocytes expressing high or low levels of SRp38. This would require oocytes that are not transcriptionally active to retain the capacity to splice mRNA. There is one example, where this has been shown to occur in platelets (Denis *et al.*, 2005). Unfortunately, the TN24 reporter cannot be used at this stage to monitor splicing efficiency, as it requires transcription to be

taking place. The fact that overexpression or depletion of SRp38 from GV-stage oocytes leads to arrest prior to GVBD, suggests an involvement of SRp38 with a cell cycle mechanism or another house-keeping process other than splicing. The only stage that was insensitive to different levels of SRp38 was the pronucleate stage, where excess of SRp38 had no effect on development to the 2-cell stage.

The fact that the effects on development are apparently independent of splicing raises the question of how development is affected when SRp38 levels are modulated. SRp38 overexpression or depletion was recently shown to interfere with normal neuronal differentiation, an effect independent of splicing (Liu and Harland, 2005). Overexpression of SRp38 blocked the activity of a protein essential for normal neurogenesis, whereas depletion of SRp38 increased neurogenesis by activation of a pathway responsible for differentiation of cells into neurons in early development. A possible explanation could be that SRp38 regulates a currently unknown pathway in oocytes or early embryos that is independent of splicing. The pathways of transcription, translation and protein synthesis however can be excluded because they were not affected in our experiments.

The depletion of other SR proteins from early embryos also inhibits normal development. When the SR protein SRp20 gene was inactivated by Cre-loxP mediated recombination, the embryos failed to develop to the blastocyst stage and died at the morula stage (Jumaa *et al.*, 1999). Another study in *Drosophila* showed that normal development was inhibited when a null mutant of

the SR protein SRp55 was generated. The development was inhibited in the first and second-instar larval stages. It was also shown in the same study that both constitutive and alternative splicing of endogenous genes were unaffected, suggesting that the SR protein is not essential for all splicing and is possibly involved in other functions (Ring and Lis, 1994).

As mentioned in the introduction, novel roles for SR proteins have been proposed. It may be that these SR proteins are essential in early development because they are required in mRNA export, translation, surveillance, or other processes not yet described.

6. Discussion

The aim of this work was to develop a splicing assay that would work on single living cells and to investigate the role and regulation of splicing in early mammalian embryos. The splicing machinery was investigated using living and fixed mouse oocytes and embryos by the use of GFP fusion proteins and imaging techniques, combined with techniques for manipulating the levels of the proteins of interest.

For many years splicing activity has been studied using splicing assays that use a particular mRNA transcript, mainly the beta-globin gene. The *in vitro* prepared transcript is introduced in cell extracts that splice the mRNA and the splicing efficiency is determined by running the mRNA products on a gel and measuring relative levels of spliced and unspliced products. Such techniques would not be possible in experiments involving early embryos, because of the requirement of large numbers of cells for preparing the cell extracts, equivalent to approximately one thousand embryos. The development of our novel fluorescent probe enabled us to measure splicing efficiency in single cells of early mammalian embryos. Two fluorescent proteins DsRed Express and EGFP were intervened by an intronic sequence that included stop codons. Upon injection of the intact vector DNA into single blastomeres of early mouse embryos, the two proteins were expressed. Splicing would excise the intron containing the stop

codons and would yield the DsRed Express-EGFP fusion protein. In the absence of splicing, only the DsRed Express would be present. Fluorescence from DsRed Express therefore reflects the transcriptional activity of the embryos, whereas green fluorescence reflects splicing activity. This technique proved to yield rapid results determined by the time taken to express the reporter. Analysis of splicing using this technique requires straight forward image analysis and there is no need for laborious biochemical protocols. The properties of the probe were tested against a number of conditions including the deletion of the 5' splice site, the use of different introns and the inhibition of U1. In all cases the data were consistent with the conclusion that the probe was a reliable reporter of splicing activity.

In the lead up to developing this splicing reporter we underwent a number of iterations before finally obtaining a combination of fluorescent proteins that behaved reliably. For purposes of clarity, these preparatory steps have not been presented in this thesis. Nevertheless, it is valuable to comment on the properties that maximise the chance of success when developing such fluorescent probes. EGFP is an obvious choice due to its high efficiency and extensive characterisation. A number of different partners were considered. CFP was a possibility, but its relative low fluorescence and the requirement for excitation at shorter wavelengths raised some concerns about long term experiments. On the other hand red fluorescent proteins allow the experimenter to avoid the use of short wavelength excitation while maintaining similar fluorescent spectra that allows it to be separated from EGFP (Clontech UK).

Initial attempts to develop the probe with an RFP proved problematic. At the time of these initial experiments the best RFP available was DsRed2, rather than DsRed Express. DsRed2 proved to be entirely unsuitable for the purposes of the splicing probe. Firstly, the time of maturation of the red fluorophore exceeded 15 hours, allowing the embryos to progress to the 4-cell stage, prior to the detection of any red fluorescence. Secondly, DsRed2 initially emits green fluorescence and only upon maturation does it yield red fluorescence. For these two reasons DsRed Express was, fortunately for this study, marketed by Clontech and was used to construct new vectors which proved ideal for the purpose of this study. DsRed Express does not fluoresce at all in the green spectrum and can mature and fold to obtain maximum fluorescence within 3 hours. These properties make it an ideal partner for EGFP. It was also important for the success of the probe that the two proteins had similar properties so that data for transcription (RFP) and splicing (EGFP) are obtained with similar kinetics.

DsRed Express as well as EGFP proteins mutated in many sites compared to their wild type counterparts form in order to yield faster maturation time, greater stability and solubility and reduced aggregation. DsRed 1 and 2 are known to form tetramers and in the longer term to form aggregates. These aggregates are not desired for reasons of toxicity and also because they complicate the fluorescent readings. Mutations inserted in DsRed Express significantly reduced the formation of such aggregates. During our experiments we could only see the formation of small aggregates at extremely high concentrations of red

fluorescence, i.e. over 4000 AUs. Therefore no experiments were performed at a concentration of red fluorescence over 2500-3000 AUs. A potential solution for solving such a problem would be the substitution of DsRed Express with DsRed Monomer, a protein recently presented by Clontech. This protein contains 45 amino acids substitutions so that it does not form any tetramers while maintaining similar spectral and maturation properties of DsRed Express. Even though DsRed Monomer would be a better candidate for such a probe it would not be advisable to perform experiments at such high concentrations, because we have proposed that the divergence in the rate of transcription and splicing seen in our studies may be due to saturation of splicing machinery.

We could demonstrate that splicing could also be performed efficiently when a number of introns were used in the probe. This allows for the substitution of the existing intron with alternative introns to allow splicing efficiency to be measured in single cells in systems where the intron consensus or splicing enhancer / silencer are important. Furthermore, the incorporation of cryptic splice sites within the fluorescent proteins may provide additional tools for monitoring splicing. These vectors could be designed in such a way that upon splicing of cryptic splice site, the fluorescence of the protein with the cryptic splice site is eliminated. Therefore, such probes would be important in studies where alternative splicing is important.

Another possibility would be to design probes that would be specific for measuring splicing activity in the presence of enhancer sequences. Our M1M2

probe and M1M2ΔE probes were a good starting point but there is no splice site competition in these probes. Therefore, the results may not reflect the splicing events occurring in a dynamic system. A suggestion of the some new probes that would allow monitoring of enhancer sequences is shown in the figure below.

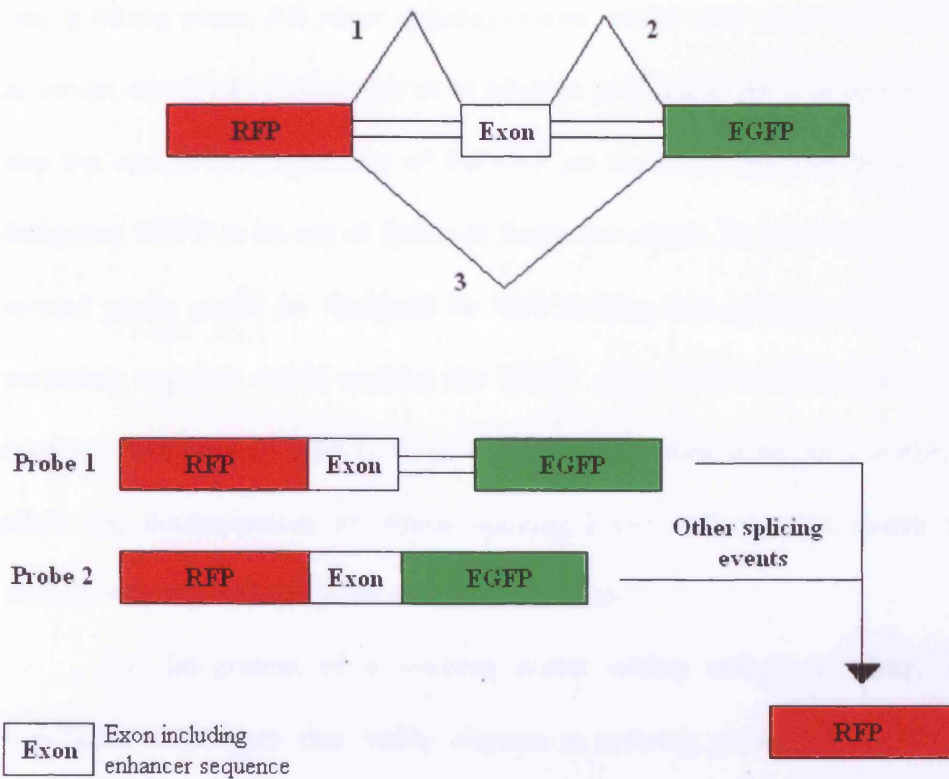


Figure 6.1. A schematic of the two probes that allow the monitoring of splicing when an enhancer sequence is used in an exon flanked by splice site that would intervene the current intron sequence. The three possible splicing events are shown with numbers. The outcomes of expression depending to the splicing events in the two probes are also shown.

A vector could be constructed as presented above in figure 6.1. The existing single intron of our current probe (e.g. TN24) could be intervened by an exon flanked by splice sites that contains the enhancer sequence. This set up of

the probe would introduce splice site competition. There are three potential splicing events that could occur shown in the figure with the corresponding number. To investigate whether splicing event one has taken place (event enhanced by the enhancer sequence), over the other splicing events, the construct would be designed in such a way that EGFP is expressed only if splicing event one is taking place. All other splicing events would only yield RFP. This probe however, would not distinguish as to whether splicing event 2 or splicing event 3 was the reason for expression of the RFP on its own. This can be achieved by designing EGFP to be out of frame in these two cases. To resolve this problem a second probe could be designed in such a way that splicing events 1 and 2 occurring together would express the EGFP. Any other combination in splicing events would yield RFP on its own. Combining the data from the 2 probes, would allow the determination of which splicing event and to what extent splicing activity was influenced by the enhancer sequence.

The integration of a splicing probe within cell lines would also be beneficial. Cell lines that stably express a splicing probe would not require transfection and therefore could be used for screening of drugs or screening of libraries to monitor splicing activity. Some preliminary experiments performed at Ian Eperon's laboratory at University of Leicester show that the expression of such an integrated probe in cell lines occurs within 12-18h, which is a short period of time compared to the 72h incubation periods essential after transient-transfection experiments.

Fluorescent proteins have not normally been used in splicing assays, however, they have been shown to be very powerful tools for localisation studies. In this work we have used an SRp38-EGFP fusion protein to address a number of questions. Firstly, to monitor the localisation of the protein during development and secondly to investigate its effect on development and splicing of early embryos. Unlike immunofluorescence, the use of the GFP-tagged proteins allows the monitoring of the protein in single living oocytes and embryos. This provides a means of studying dynamic events in relation to the developmental processes that the proteins under examination may be involved. In addition, possible artefacts produced by the fixing process during immunofluorescence can be avoided by the use of GFP technology.

In order to investigate SRp38 in live cells, SRp38 was cloned into pEGFP-N1. The N-terminal of the SRp38 is involved in RNA binding interactions, whereas the C-terminal of SRp38 possesses the RS domain involved in protein-protein interactions. Therefore, EGFP is expressed downstream of SRp38's RS domain. EGFP fusion proteins have been successfully used for monitoring of protein function and localisation experiments, without interfering with the protein's properties. Nevertheless, it could be argued that EGFP may interfere with the RS domain of SRp38. Our immunofluorescence data correlates very closely with the localisation data obtained by the SRp38-EGFP protein. The immunofluorescence data therefore could confirm that the distribution of SRp38-EGFP was not affected by the EGFP tag. The effects of the overexpression of

SRp38 were quite surprising. SRp38 has been characterised as a potent inhibitor of splicing especially in its dephosphorylated form (Shin and Manley, 2002). The phosphorylated form of the protein should also inhibit splicing to a less extent (Shin and Manley, 2002). However, we could not show with our probe that splicing was inhibited in early embryos. The fact that overexpression of the protein inhibited the development of GV-stage oocytes (a stage in which no transcription is taking place), as well as previous data of dysregulation of neuronal differentiation (Liu and Harland, 2005), suggest that the protein may be involved in processes other than splicing. This finding is not surprising. In the past, SR proteins were believed to be splicing regulators. However recent studies have implicated them in mRNA translation, mRNA export and mRNA surveillance and quality control. Our data suggests that SRp38 might interfere with mechanisms necessary for oocyte maturation and early embryo development. It is also important to mention that the use of a new fluorescent probe, such as the SRp38-EGFP needs extensive characterisation to ensure that the signal is a true reflection of the behaviour of the probe and that the fluorophore used does not interfere with the normal progression of the cell cycle in a non-specific way. Comparison of immunofluorescence and live imaging has revealed that our SRp38-EGFP fusion protein presented the same pattern of localisation. The fact that EGFP expressed in the cell did not inhibit normal development suggests that the findings of our experiments reveal the effects of SRp38 rather than the effects of EGFP.

Depletion of a protein can also yield useful information on the importance of such a protein during these early stages in development. There are two main techniques widely used for the depletion of proteins. The method of RNA interference (RNAi) involves the injection of RNA with complementary sequence to the protein's in question mRNA. Upon formation of the mRNA duplex, the mRNA is degraded resulting in depletion of the protein. A second method of protein depletion involves the use of a morpholino oligo. In a similar principle as with RNAi, a cDNA oligonucleotide, complementary to the target mRNA sequence is injected in the cell. In this case the mRNA is not degraded, but the morpholino molecule inhibits the binding of the translational machinery onto the mRNA molecule thus inhibiting its expression. The use of the morpholino approach in this study was partially successful in the case of SRp38. Depletion caused the arrest of the depleted embryos at the 2-cell stage. This finding was not surprising due to the fact that zygotic genome activation occurs at the same time that the protein was depleted from the cell, around the 2-cell stage.

Recent micro-array studies have revealed that the first genes expressed during zygotic genome activation include transcription and splicing factors. In particular, three SR proteins genes, *Srfs1*, *Srfs5*, *Srfs7* have been identified by transcript profiling studies to be expressed from the late 1-cell stage (Zeng *et al.*, 2004; Hamatani *et al.*, 2004a). Depletion or overexpression of SRp38 did not inhibit splicing activity.

In HeLa cells splicing was shown to be regulated by a nuclear PIP₂ pool that localises with SC35. Osbourne and colleagues (2001) performed experiments in which splicing was inhibited in cell extracts depleted of PIP₂. However the protocols used for the depletion of PIP₂ could potentially eliminate other factors and one of those could be SC35 since it co-localised with PIP₂. SC35 is known to be a potent activator of splicing, and its depletion from the cell could have detrimental effects to the cell splicing machinery. Our immunofluorescence data presents the SC35 arranging in speckles during the late 2-cell stage. However, the PIP₂ is presented with a uniform distribution at the late 2-cell stage and arranges in speckles from the 4-cell stage onwards. This data therefore suggests that it is not nuclear PIP₂ that regulates splicing in early development since its organisation in the speckle is later than that seen by SC35.

Splicing, as mentioned earlier, is an essential process for gene expression and therefore it is very carefully regulated during early development. Splicing stops when transcription stops when the oocytes are fully grown. In order to begin at the onset of transcription, maternal splicing factors must remain to perform this function. It is not known whether these proteins are stored, or whether new translation is necessary. However, recent RNA profiling studies show that SR proteins are expressed as early as the late 1-cell stage (Zeng *et al.*, 2004; Hamatani *et al.*, 2004a). This suggests that maternal splicing factors support splicing at this very early stages of development, for the expression of splicing factors required

for development. The expression of these embryonic splicing factors may then be used for splicing of genes expressed from the mid-late 2-cell stage onwards.

This thesis presents a novel fluorescent reporter that allows the monitoring of splicing activity in early embryos. This reporter was used as a valuable tool in this thesis, to investigate splicing activity in early mammalian development. It was demonstrated that the embryos are capable to splice from the pronucleus stage, even without the appearance of spliceosomes in the nuclei. Finally, it was presented the SR protein SRp38 plays an important role in early mammalian development, in splicing and possibly other processes.

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