The effect of nutrients upon the activity of SR proteins

Callee McConnell Walsh
West Virginia University

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The Effect of Nutrients upon the Activity of SR Proteins

Callee McConnell Walsh

Dissertation submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology

Lisa Salati, Ph.D., Chair
Stephen Graber, Ph.D.
Thomas Elliott, Ph.D.
Max Sokolov, Ph.D.
Aaron Timperman, Ph.D.

Department of Biochemistry

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ABSTRACT

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Callee McConnell Walsh

RNA splicing is an important component of gene expression that contributes immensely to the biological complexity of higher eukaryotes. The regulation of splicing by external stimuli, such as nutrients, is poorly understood, however. The experiments contained herein sought to understand how the activity of splicing factors called SR proteins is regulated by insulin and polyunsaturated fatty acids in the liver using the model mRNA glucose-6-phosphate dehydrogenase (G6PD). RNA affinity and chromatin immunoprecipitation experiments demonstrated that refeeding of rodents stimulates the binding of SR proteins to the splicing regulatory element of G6PD; and this was especially relevant for SRp20. In vitro and in vivo splicing assays demonstrated that SRp20 is relevant for the splicing of G6PD. Additionally, analysis of SR proteins in hepatocytes determined that insulin stimulates the amount of phosphorylated SRp20 in the nucleus by 13-fold while the polyunsaturated fatty acid, arachidonic acid, attenuated this by 80%. A similar pattern was observed for SRp30 a/b and SRp40. These data suggest that the increase in binding activity of SRp20 during refeeding is stimulated by the increase in phosphorylation via insulin. These findings demonstrate that the activity of SR proteins changes in response to nutritional status and can ultimately impact the splicing of target mRNAs.
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Clk/Sty kinase</td>
<td>Cdc2-like kinase/serine/threonine/tyrosine kinase</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain of RNA polymerase II</td>
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<tr>
<td>ESE</td>
<td>Exonic splicing enhancer</td>
</tr>
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<td>ESS</td>
<td>Exonic splicing silencer</td>
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<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HnRNP</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMD</td>
<td>Nonsense-mediated decay</td>
</tr>
<tr>
<td>nPTB</td>
<td>Neuronal polypyrimidine tract binding protein</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>Pol II</td>
<td>RNA Polymerase II</td>
</tr>
<tr>
<td>Poly(A) tail</td>
<td>Polyadenylated tail</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>PP2</td>
<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>PTB</td>
<td>Polypyrimidine tract binding protein</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA-recognition motif</td>
</tr>
<tr>
<td>RS domain</td>
<td>Serine-arginine rich domain</td>
</tr>
<tr>
<td>SnRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SRPK</td>
<td>SR protein kinase</td>
</tr>
<tr>
<td>SR protein</td>
<td>Serine and arginine rich protein</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box binding protein</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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CHAPTER 1

Introduction

The process of gene expression involves coordination of many different processes within different regions of the cell. Beginning with transcription to pre-mRNA processing to finally translation, the many processes are complex, intertwined, and elegantly coordinated. The discovery of split genes occurred in 1977 (1), revealing the importance of splicing, the process of intron removal and exon ligation, in gene expression. Most markedly, since the sequence of the human genome was completed in 2003, it is realized that splicing expands the protein diversity gleaned from our relatively small number of genes, and recent reports indicate that greater than 90% of our genes undergo alternative splicing (2, 3). Regulation of splicing is, thus far, a poorly understood area of research that is just beginning to be explored. As numerous recent reports indicate that splicing is interconnected to other aspects of gene processing and expression, it is becoming increasingly important to understand the regulatory processes of splicing.

Transcription

The process of transcription in eukaryotes is complex and is regulated at many stages. General transcription factors assist RNA polymerase II (Pol II) to bind to promoters and initiate transcription at the transcription start site, which is demarcated by the initiator motif, YYANWYY, in humans (W=T/A) (4, 5). The core promoter recognition motif is the TATA box, 25-33 base pairs upstream of the start site, which is recognized by the general transcription factor TBP (TATA-box binding protein), a component of TFIID (5). Upon the binding of TBP to the TATA box, TFIIB binds followed by TFIIF, Pol II, TFIIE, and TFIIH, which completes the formation of the preinitiation complex (5). TFIIH has helicase activity to unwind the DNA strands at the transcription start site and allows transcription to proceed. This protein also phosphorylates the C-terminal domain (CTD) of Pol II to allow transcriptional elongation to begin. Many other factors regulate the transcription process beyond these general
transcription factors, however. Transcription factor binding sites and the transcription factors that bind to them regulate the recruitment of Pol II to promoters and transcription start sites. Also, transcriptional repressor sequences and repressor proteins can inhibit this process in response to appropriate stimuli. Transcription factor binding sites are often located long distances upstream or downstream from the promoters they regulate (6). Additional regulatory control of transcription is provided by histones to which chromatin is bound. Specific combinations of acetylation and methylation of residues within histones 3 and 4 are associated with euchromatin, which allows greater access of Pol II to promoters. Other discrete combinations of mainly methylated and some acetylated residues are associated with transcriptionally inactive heterochromatin (7).

Pol II is a multimeric enzyme that is unique among other RNA polymerases in the cell. Each polymerase transcribes different classes of RNAs with Pol II transcribing mRNAs and most of the small nuclear RNAs that are components of small nuclear ribonucleoproteins (snRNPs) involved in splicing. Pol I transcribes rRNAs, and Pol III transcribes mainly tRNAs. Of the three RNA polymerases, only Pol II contains the unique domain, the CTD. In mammals, this domain consists of 52 repeats of the sequence YSPTSPS of which 21 of these repeats diverge to some extent from the consensus. Although 5 of the 7 residues within the repeat are subject to phosphorylation, the serines located at position 2 and 5 are the main targets of regulation. Serine 5 is associated with transcription initiation and promoter clearance and serine 2 phosphorylation is important for elongation of transcription (8, 9). Several of the mRNA processing factors, such as the enzymes involved in 5’ cap and poly (A) tail formation, are directly recruited to the CTD of Pol II (10), which allows for co-transcriptional processing as soon as the nascent pre-mRNA emerges from the enzyme. Therefore, the unique CTD of Pol II is responsible for initiating the 5’ cap and poly (A) tail (polyadenylated tail) upon mRNAs, which distinguishes them from other cellular RNAs.

**Pre-mRNA processing, export, and translation**

As the pre-mRNA is being described, it is modified through several processing steps. These include 5’ capping, removal of introns in splicing, and cleavage and
addition of the poly(A) tail to the 3’ end of the pre-mRNA. These processing steps increase the stability of the mRNA, properly order the sequences of exons in the mRNA, and increase its ability to be exported from the nucleus to the cytoplasm for translation.

The first step in pre-mRNA processing is capping of the 5’ end of the pre-mRNA after 25-30 nucleotides have emerged from Pol II. Three enzymatic activities are necessary including a tri-phosphatase, a guanyl transferase, and a methyl transferase, which are associated with the CTD. Their activity converts the pppA 5’ terminus to a 7-methyl guanosine cap (11). The cap is necessary for stability, splicing, polyadenylation, export, quality control, and translation of the mature mRNA (12). The cap recruits the cap binding protein, which is a heterodimer consisting of CBP-80 and CBP-20. These binding proteins initiate cap-dependent translation early in the translation process and are important for the pioneer round of translation, being subsequently replaced by translation factors during steady state translation (13).

The next processing activity upon the pre-mRNA is removal of intronic sequences from the protein-coding exonic sequences, which is termed mRNA splicing. Although the two transesterification reactions required for splicing are catalyzed completely by the RNA itself, several ribonucleoprotein complexes are necessary for the structural organization of the pre-mRNA to achieve a catalytically active intron. Sequences within the introns and exons of RNA provide docking sites for many of the ribonucleoproteins and proteins involved in the splicing process. The most basic sequence requirements for splicing are found within introns, which include the 5’ splice site (GU), the branch point (A), the polypyrimidine tract, and the 3’ splice site, AG (Figure 1; 14). These sequences recruit snRNPs and other accessory proteins to the intron to form the protein complex called the spliceosome. Specifically, U2AF$^{65}$ binds to the polypyrimidine tract, U2AF$^{35}$ binds to the 3’ splice site, SF1 binds to the branch point, and U1 snRNP binds to the 5’ splice site through direct RNA-RNA and protein-RNA interactions. This initial assembly of proteins constitutes the E complex or early complex of the spliceosome. Following formation of the E complex, U2 snRNP interacts with the branch point A in an ATP-dependent manner and forms the A complex of proteins. The B complex is formed next by the addition of the tri-snRNP containing U4/5/6 snRNPs. This complex of proteins rearranges the RNA in order for the first transesterification reaction to occur via
nucleophilic attack of the 5’ splice site by the branch point A. Next, the 5’ splice site attacks the 3’ splice site resulting in formation of the intron as a free lariat and the splicing together of adjacent exons. This is an overly simplified description of the splicing process because the spliceosome is estimated to contain over 100 proteins (15), and the activity as well as the coordination of all of the proteins within the spliceosome is poorly understood. Among the components of this complex, the activity of a few protein families is emerging. Helicases are an important component of the spliceosome for their function in rearranging protein-protein and protein-RNA complexes, allowing dynamic changes in the spliceosome. The main family members involved include Sub2, Prp5, Prp28, Brr2, Prp2, Prp16, Prp22, and Prp43 (16). Also, SR
proteins (serine-arginine rich proteins) and hnRNPs (heterogeneous ribonucleoproteins) are important mediators of the splicing process with regulation of spliceosome formation being their best-characterized function. Proper removal of intervening introns consequently deposits a complex of proteins 20-24 nucleotides upstream of the exon-exon junction, which is called the exon junction complex (17, 18). The exon junction complex is important for quality control processes and export of the mRNA.

Polyadenylation is the final processing step upon the mRNA. The sequence of most mRNAs contains a polyadenylation signal (AAUAAA) and a downstream element consists of either a G/U or a U-rich sequence (19). Both are located within the 3’ untranslated region. The cleavage and polyadenylation specificity factor first binds to the 5’ poly(A) signal, which then recruits the cleavage stimulatory factor, cleavage factor I, cleavage factor II, and finally poly(A) polymerase (19). Upon formation of this protein complex, the mRNA is cleaved between the poly(A) signal and downstream element and poly(A) polymerase proceeds to add 200-250 A residues (19). Poly(A) binding proteins then bind to the poly(A) tail, which stabilize the mRNA through interaction with the cap-binding complex, inhibiting the access of RNases. Additionally, alternative polyadenylation sites exist and are a common mechanism to enhance protein diversity (20).

RNA export into the cytoplasm depends upon the sequential deposition of several proteins during capping, splicing, and polyadenylation processes. Both the 5’ cap and the exon junction complexes, placed upon the mRNA during splicing, are necessary for recruitment of the transcription export factor called TREX and its component ALY (21-23). The poly(A) tail recruits nucleoporin proteins that are components of the nuclear pore complex (24). Components of TREX interact with TAP and several other proteins, which bind the mRNA to the export receptor (25). The export receptor interacts with phenylalanine and glycine rich domains within the nuclear pore complex, and the mRNA is exported via facilitated diffusion (26). After a series of successive removals of proteins performed by DEAD-box proteins, the cap binding protein is replaced by the translation initiation factor, eIF4E.

Translation of the mRNA is an extremely complex process with many proteins involved. As this review focuses upon mRNA processing and, specifically, splicing,
translation will only be described in general as is sufficient to place it in the context of the impact of splicing factors upon the translation process. Translation is initiated with the binding of the preinitiation complex. This complex scans the 5’ UTR of the mRNA to identify the AUG codon (25). Translation ensues as the mRNA is held in a circular formation by a linkage between the 5’ cap and poly(A) tail. Peptide bond formation initiates at this point with amino acids being delivered via tRNA and continues until the stop codon is met (27, 28). At this point, release factors recognize the stop codon and promote cleavage of the peptidyl-tRNA through GTP hydrolysis, and this releases the new protein. A single mRNA can be translated simultaneously by several ribosomes at once, but after a period of time, the mRNA integrity is undermined by deadenylases and de-capping enzymes, making the mRNA susceptible to nucleases. This ceases the translation process.

RNA Quality Control

At several points during mRNA processing, quality control mechanisms remove improperly processed mRNAs. The nuclear surveillance pathway removes aberrant transcripts through degradation in the nuclear exosome. This pathway detects and degrades unspliced mRNAs, transcripts with aberrant poly(A) tails, and transcripts that fail to acquire the proper RNA-binding proteins throughout transcription and splicing (29, 30). It also eliminates the intronic lariats that arise from the splicing process after they are first processed by the debranching enzyme.

Another type of surveillance pathway is nonsense-mediated decay (NMD), which detects the presence of premature termination codons in spliced transcripts. Premature termination codons can arise through several means including errors in transcription, frame shift mutations, programmed DNA rearrangements, which is common in T-cell receptors, and also aberrant splicing (31). Transcripts containing premature termination codons, if translated, could be extremely deleterious to the cell, creating truncated proteins with toxic gain-of or loss-of function characteristics; therefore, rapid removal of such transcripts is essential. The exon junction complex is instrumental in the NMD pathway because it marks exon-exon junctions and also contains protein components of
the NMD pathway (Upf2 and Upf3). Upon export of a transcript from the nucleus, a pioneering round of translation is performed that identifies premature termination codons located 50 nucleotides upstream of the last exon junction complex (32). This triggers the NMD pathway to activate via the interaction of Upf1 with Upf2 and Upf3 (32). The mRNA is degraded from the 5’ end by the Xrn1 exonucleolytic activity after decapping or the 3’ end by the cytoplasmic exosome after deadenylation (33, 34).

**Importance of Splicing**

There are two types of pre-mRNA splicing: constitutive splicing and alternative splicing. Constitutive splicing is the removal of intervening introns and subsequent ligation of exons that are always present within a particular transcript and not subject to regulated inclusion. Alternative splicing is the process by which exons and introns are included in the transcript in a regulated manner. The types of alternative splicing are exon skipping, mutually exclusive inclusion of exons, alternative 5’ splice sites, alternative 3’ splice sites, and intron retention (Figure 2; 14). Alternative and constitutive splicing use the same mechanisms of splicing; the only difference is that alternative splicing is subject to greater regulatory variability (35).

Alternative splicing increases the number of proteins that arise from the genome (36). The human genome contains approximately 23,000 genes, while the size of the proteome is estimated to be upwards of 90,000 different proteins, which equates to a protein to gene ratio of four to one (37). The almost unlimited combination of sequences that alternative splicing produces has expanded the proteome to, thus far, an immeasurable level in humans. These data highlight just how prevalent and how important alternative splicing is in human gene expression, creating multiple mRNAs and proteins from a single gene (36). In fact, recent research suggests that greater than 90% of our genes are alternatively spliced (2, 3). This percentage stands in stark contrast to the protein to gene ratio in the fly and worm where alternative splicing is less prevalent and the protein to gene ratio is near one to one (37).
Figure 2. Types of Alternative Splicing. Adapted from Cartegni et al., 2002.
Alternative splicing also enhances the diversity of proteins in the human proteome. Changes in the primary sequence through alternative splicing have consequences for the function of the resulting protein. Alternative splicing can change the binding properties, the post-translational modifications, the catalytic activity, localization, and many more properties of the translated proteins (38). Alternative splicing also can regulate the amount of the protein that is expressed through inclusion of exons or introns containing premature termination codons, and this subjects the mRNA to NMD. The exquisite control of protein activity through alternative splicing is important to many processes such as development and response to external stimuli to maintain homeostasis.

The process of alternative splicing is the result of several factors, which is termed combinatorial control. Many factors affect exon recognition and formation of the spliceosome. These factors include sequences within exons and introns, the length of introns and exons, the speed of transcription, the relative amounts of positive and negative trans factors within a certain cell type, the activity of these trans factors, and the position of nucleosomes with respect to splice sites. In addition, secondary structure of the RNA may also exert regulatory control upon the splicing process (39). Thus, many levels of complexity exist in the processes of constitutive and alternative splicing, and many of these topics will be addressed in the following sections.

**Splicing Regulatory Sequences**

Sequences within the pre-mRNA provide binding sites for splicing factors, which provide regulatory control over splicing. As discussed above, consensus sequences within the intron serve as binding sites for snRNPs. Many additional sequences within exons and introns are also necessary for proper recognition of exons in constitutive splicing and regulated inclusion of exons and introns in alternative splicing. Exonic splicing silencers (ESSs) are located within exons and generally inhibit the splicing process. Exonic splicing enhancers (ESEs) are sequences within exons that promote splicing and exon recognition. ESEs perform this task by providing binding sites for a
family of proteins called SR proteins. The canonical family of SR proteins includes 9G8, SRp20, SC35, ASF/SF2, SRp40, SRp55, SRp75, and Tra2β. Some ESEs show specificity in the binding of a particular SR protein. For example, the ESE within exon 3 of the Tat gene promotes association of the SR protein ASF/SF2 but not SC35 (40). On the other hand, some ESEs have less specificity with respect to SR protein binding such as in the case of β-globin in which both SF2/ASF and SC35 can enhance splicing (40).

ESEs and SR proteins promote inclusion of a particular sequence, but they serve slightly different purposes in constitutive and alternative splicing. In the splicing of constitutive exons, ESEs and SR proteins play a role in distinguishing exons from the surrounding intronic sequences, a process called exon definition (41). The binding of SR proteins to ESEs is essential for stimulating inclusion of alternatively spliced exons, which tend to have weak intronic splicing sequences such as nonconsensus 5’ and 3’ splice sites and polypyrimidine tracts (42). In these cases, these splicing signals would be too weak to recruit U1 snRNP, U2AF₃₅, or U2AF₆₅ alone, and splicing would not occur or would be less efficient. In support of this hypothesis, a recent study showed that a minigene that required ASF/SF2 for splicing could splice independently of ASF/SF2 when its 5’ splice site was mutated to more closely resemble the consensus binding site for U6 snRNP (43). While, there is no relationship between splice site strength and the frequency of ESEs in constitutively spliced exons, the authors suggest that a relationship may exist such that weak splice sites may be associated with stronger rather than multiple ESEs (44). Additionally, alternative splice sites have fewer ESE motifs than constitutive splice sites (44), which presumably is the reason for their irregular inclusion.

Efforts to identify and characterize ESEs have produced vague results. One reason for the difficulty in identifying ESEs is that several SR proteins lack fidelity in sequence binding. ESEs can be purine (A and G) rich, can contain the consensus sequence (GAR)ₙ, or can be AC-rich (14, 45-47). Additionally, ESEs are enriched in the regions of exons close to the 3’ and 5’ splice sites of intervening introns (46) where these sequences would have a higher propensity for the interaction of an SR protein with the basal splicing machinery (snRNPs). Several groups have developed computer programs to predict ESEs based upon several parameters such as proximity to splice sites, strength of splice sites, and binding to SR proteins. Another method to define binding sites is
called SELEX (selective evolution of ligands by exponential enrichment). This is a purely \textit{in vitro} method in which random sequences that are bound by the protein of interest are captured and enriched with PCR. These analyses have provided degenerate hexamers or octamers that must be functionally examined to determine their actual role in splicing.

The recent advent of high throughput sequencing, however, has created an opportunity to better define the sequences to which SR proteins bind. The first definition of a global consensus-binding site for an SR protein was recently determined utilizing high throughput sequencing technology. Using \textit{in vivo} crosslinking followed by immunoprecipitation and sequencing of the bound RNA, the Cacerces group identified the global binding sequence of ASF/SF2 to be TGAT/AGAA, which is somewhat different from consensus binding sites identified with traditional methods (48). This technique is superior to \textit{in vitro} methods such as SELEX because the interaction of the RNA-binding protein and its target RNA is captured with the crosslinking step in intact cells as splicing is occurring. The crosslinking step creates stable covalent bonds between the protein and RNA, which prevents rearrangement and loss of the interaction that can potentially occur with immunoprecipitation without a prior crosslinking step. High throughput sequencing enables the sequencing of tens of millions of bases within days (49). Overall, \textit{in vivo} crosslinking immunoprecipitation coupled with high throughput sequencing is a promising technique for defining consensus binding sites of RNA-binding proteins, which has historically been a difficult task.

ESSs are involved in constitutive and alternative splicing events, but they have received much less attention than ESEs. ESSs in general inhibit the inclusion of the exon in which they’re located and have major roles in alternative splicing of exons by inhibiting inclusion in a regulated manner. By screening human genomic DNA libraries for splicing inhibitory sequences, Fairbrother and Chasin (50) concluded that ESSs and splicing inhibition may be quite common in the human genome. They found that 1 in 3 randomly chosen sequences from human DNA inhibited splicing, while only 1 in 27 sequences from E. coli genomic DNA was inhibitory (50). Several studies have attempted to characterize ESSs in a global manner. Wang et al. (51) generated random decamers, inserted them into a splicing reporter minigene, and measured the resulting
mRNA products after transfection. Upon sequence comparison, they identified 7 clusters of ESSs that contained 6 nucleotides each. These hexamers consisted of mainly U (38%) and G (36%), which has also been found by others (52). Others have characterized ESSs as having a high abundance of pyrimidines (50). Additionally, ESS hexamers were identified more often in strong exons than weak exons that are constitutively spliced and more often in alternative exons that are skipped than constitutive exons (51). In addition to the nucleotide sequence, the position of a sequence within an exon may determine its effect on splicing as a particular sequence can both enhance and repress depending on its location (53). Additional observations include a higher frequency of these inhibitory sequences found in pseudoexons than constitutively spliced exons (51). Pseudoexons are found within introns and resemble authentic exons in that they contain 5’ and 3’ decoy splice sites that match consensus sequences, but they are rarely spliced (54). Therefore, one role of silencer sequences is thought to be repression of pseudoexon splicing (51, 55).

Besides ESEs and ESSs, additional sequences within the intron can impact the splicing process. ISEs (intronic splicing enhancers) are located within introns and enhance recognition of particular sequences, and ISSs (intronic splicing silencers) block recognition of particular sequences. A well-studied ISS is an inhibitory sequence that is associated with some polypyrimidine tracts. Although the polypyrimidine tract provides a binding site for U2AF65 and promotes recognition of other intronic splice sites by U2 snRNP, as discussed above, this sequence can also inhibit intron recognition. This occurs through its recognition by polypyrimidine tract binding protein (PTB), which binds to the consensus motif, CUCUCU that is surrounded by pyrimidines. PTB is an active repressor of weak exons that may function by competing with U2AF65 for binding to the intron (52). In a specific example, this ISS and PTB affect alternative splicing of c-src pre-mRNA. In this pre-mRNA, PTB binds to ISS sequences within the upstream and downstream introns surrounding the regulated exon called N1 (56, 57). This binding is thought to “loop out” the N1 exon and result in exon skipping. Interestingly, this exon is only skipped in non-neuronal tissues. The reason for this tissue specific splicing is the expression of a neural version of PTB called nPTB (neuronal PTB) in the brain. nPTB is deficient in binding to the upstream ISS; therefore, the exon is included in the final transcript (58).
ISE sequences and the proteins that bind to them enhance the inclusion of alternative exons. One of the best examples of a regulatory ISE is UGCAUG which provides a binding site for FOX-1 and 2 proteins in transcripts from neural tissues, in particular (reviewed in 59). This ISE is often located up to 400 nucleotides downstream from the regulated exon but can enhance inclusion from distances as great as 1000 base pairs downstream from the regulated exon (60, 61). This ISE is necessary for inclusion of the N30 exon in myosin heavy chain B in neural tissue; in non-neural tissue this exon is skipped. It was shown that the ISE substitutes for a suboptimal polypurin tract within the intron downstream of the regulated exon, as strengthening the polypurin tract abolished the need for this particular ISE (61).

The location of intronic regulatory elements within either the upstream or downstream intron has an impact upon inclusion of the intervening exon. An example of this effect of intronic location upon alternative splicing is observed with the splicing protein called NOVA. Recent experiments in which in vivo crosslinking, immunoprecipitation of NOVA protein, and sequencing of the transcripts to which NOVA was bound were performed and produced several interesting observations. Binding sites for NOVA in an intron upstream of a regulated exon (near the 3’ splice site) inhibited inclusion of the exon in the final transcript. Conversely, binding sites for NOVA in the downstream intron, near the 5’ splice site, enhanced inclusion of the alternatively spliced exon in the final transcript (62). This effect is due to competition for splicing factors where upstream enhancers outcompete downstream splice sites for splicing factors, leading to exon skipping. Conversely, an ISE located in the downstream intron relieves competition for splicing factors from the upstream splice sites, leading to exon inclusion (reviewed in 59). The NOVA splicing factor exemplifies the extreme importance of the location of intronic splicing sequences upon the final spliced product.

**HnRNPs in splicing regulation**

HnRNPs are a family of proteins with diverse roles in RNA metabolism. These proteins are involved in RNA transport, transcription, polyadenylation, RNA decay, RNA localization, and splicing (63, 64). HnRNP A1 is the best-characterized hnRNP with a
role in repression of splicing. It can bind to ESS as well as ISS sequences. As an example, hnRNP A1 binds to the ESS within HIV Tat exon 3, causing nucleation of several hnRNP A1 proteins, preventing recognition of the exon by SR proteins, and resulting in exclusion of the exon (65).

HnRNP A1 can also inhibit inclusion of exons by binding to nearby introns. An example of this exists for the hnRNP A1 pre-mRNA itself. Through binding to ISS sequences in the intron and looping out of an alternative exon, this alternative exon is excluded (66). These and many other examples indicate that hnRNPs inhibit the splicing process by blocking recognition of ESEs by SR proteins and/or by directly interfering with the recognition of splice sites by snRNPs. It should be noted, however, that in addition to their function in inhibition of splicing, some hnRNPs are known to enhance recognition of splice sites (e.g. 67).

**SR proteins and splicing mechanisms**

The family of SR proteins has roles in both constitutive and alternative splicing. SR proteins are expressed in all tissues, but the expression level varies by tissue (68, 69). Tissue-specific differences in expression levels of SR proteins (and hnRNPs) are thought to contribute to differences in splicing patterns between tissues. Although initial *in vitro* studies indicated that SR proteins were functionally redundant, recent studies, in which individual SR proteins have been depleted, indicate that SR proteins are functionally specific (70).

Two domains define SR proteins. They contain an RNA recognition motif (RRM) at the N-terminus of which SR proteins contain one or two. They also contain a serine-arginine rich domain (RS domain) at the C-terminus for which SR proteins are named. The RS domain contains multiple arginine-serine dipeptides; for example the RS domain of ASF/SF2 contains 14 RS dipeptides plus 5 additional serines that are preceded by amino acids other than arginine (Figure 3). The serines within the RS domain are targets of several kinases that modulate the activity of SR proteins. The RRM is necessary for the SR protein to recognize and bind to its cognate RNA sequence, and it provides specificity in binding of the SR protein as substitution of an RRM from one SR
Figure 3. Domains of SR proteins. RRM = RNA recognition motif, RS = arginine-serine rich domain, Z = zinc knuckle. Adapted from Graveley, 2000 and Ngo et al., 2005.
protein with that from another changes its RNA-binding pattern to that of the substituted RRM \textit{in vivo} (71). The RRM is dispensable in the splicing process, however, and is merely required for targeting of the SR protein to the proper region for splicing (72). The RS domain is necessary for the activity of SR proteins in the splicing process. There are two main models by which SR proteins enhance the splicing process, in general, including the recruitment model and the inhibitor model (35, 70).

In the recruitment model, SR proteins bind to RNA sequences, which enhances the binding of snRNPs and encourages spliceosome formation at splice sites. The RS domain is integral to the interaction of SR proteins with snRNPs. \textit{In vitro} studies with purified proteins indicate that the SR proteins, ASF/SF2 and SC35, bind to the 70 kDa protein of U1 snRNP and to U2AF^{35}, and this interaction occurs via the RS domain (73-75). The interaction of ASF/SF2 and the U1 70 kDa protein also occurs \textit{in vivo} (71), but currently debate exists concerning whether all SR proteins interact with U1 snRNP (cf. 71, 74). These protein-protein interactions are thought to stabilize the pre-spliceosomal complexes, define the 5’ and 3’ splice sites, and help recruit other members of the spliceosome (73, 74, 76). Additionally, phosphorylation of the RS domain enhances the interaction of SR proteins with other splicing factors such as U1 snRNP (75, 77).

The recruitment model of splicing is integral to the process of exon definition of constitutive exons. In higher eukaryotes, pre-mRNAs typically contain long introns separated by short exons; thus for proper splicing to occur, the exons must be distinguished from the intronic sequences in a process called “exon definition” (41). SR proteins are important for exon definition in which the binding of SR proteins to ESEs within exons distinguishes exons from intronic sequences. This enables the recruitment of U2AF^{35}, U2AF^{65}, and U2 snRNP to the upstream 3’ splice site and U1 snRNP to the downstream 5’ splice site leading to spliceosome formation (41).

In another version of the recruitment model, the RS domain of SR proteins binds directly to intronic RNA at the 5’ splice site and the branch point. This interaction is thought to stabilize the charge repulsion at the RNA-RNA interactions between U1 and U2 snRNPs and pre-mRNA (78-80). In contrast to the two mechanisms of recruitment that require RS domains, however, recent work indicates that RS-domain independent
splicing enhancement is possible (81); therefore, the role of the RS domain in recruitment of snRNPs may be specific to each splicing event and the SR protein involved.

In the inhibitor model of splicing enhancement, SR proteins antagonize the effects of ESS sequences and inhibitory splicing proteins upon the splicing process. ESS sequences are very common throughout the human genome; in fact, the default may be splicing inhibition (50). As discussed above, hnRNPs are a family of RNA-binding proteins that, in general, are inhibitory to the splicing process through their binding to ESSs. They inhibit splicing by preventing the binding of U1 and U2 snRNPs to intronic consensus sequences. Several reports indicate that the binding of SR proteins to ESEs can counteract the inhibitory effects of ESSs when the two sequences are closely juxtaposed (65, 82, 83). As an example of this type of interaction, the Krainer group determined that the binding of ASF/SF2 to an ESE within exon 3 of the Tat pre-mRNA blocks the binding of hnRNP A1 to this same region of the exon. The binding of ASF/SF2 and prevention of hnRNP A1 binding enables exon recognition to proceed (65).

Although SR proteins are most known for their ability to enhance splicing, there are examples where SR proteins can antagonize the splicing process. For example, SRp20 enhances and ASF/SF2 antagonizes exon inclusion in SRp20 pre-mRNA to regulate expression of SRp20 protein (84, 85).

**Regulation of SR protein activity**

The ability of SR proteins to impact the splicing process depends on their ability to bind to target transcripts, which is generally termed their activity. The activity of SR proteins in the splicing process is regulated by several factors. As most SR proteins shuttle between the nucleoplasm, cytoplasm, and storage sites within the nucleus, one of the main ways that activity is regulated is by the location of the SR protein intracellularly. These shuttling processes and interactions with other splicing proteins are regulated by phosphorylation of the RS domain by the action of several different kinases and phosphatases. An additional means of regulation of SR protein activity is through changes in expression, which can occur through transcriptional and post-transcriptional mechanisms.
SR protein kinases

The splicing activity of SR proteins is regulated by their location within the cell. SR proteins shuttle between three different cellular compartments. They localize to nuclear regions called speckles when they are inactive in splicing, such as when transcription is inhibited (86). From speckles, the SR proteins can move to the nucleoplasm where they interact with actively transcribed pre-mRNA (86). They can then remain bound to the mRNA following splicing catalysis and can be exported with the mRNA to the cytosol (87); only SC35 doesn’t shuttle between the nucleus and cytosol (88). They can then be imported back into the nucleus to speckles (Figure 4). Phosphorylation and dephosphorylation of the RS domain is the main mechanism by which the movement of SR proteins between the cytosol, nuclear speckle, and nucleoplasm is regulated. An example of this regulation is exhibited in muscle cells in response to insulin treatment. The Cooper lab recently demonstrated that Akt2, activated by insulin, phosphorylates and activates a kinase called Clk/Sty, which is a known SR protein kinase. This kinase phosphorylates several SR proteins, which causes them to bind to the target pre-mRNA, PKCβII. The binding of SR proteins causes inclusion of the PKCβII exon that results in the isoform of PKCβII that enhances glucose uptake (89). This example demonstrates how signaling pathways initiate phosphorylation events that enhance the activity of SR proteins upon target transcripts. It should be noted, however, that this pathway by which SR proteins shuttle through cellular compartments through changes in phosphorylation is the combination of the work from several groups and has not been demonstrated in total for an individual SR protein.

SR proteins are phosphorylated by several kinases. SR protein kinases (SRPKs) are one family that consists of SRPK1 and 2, and they can be located in the cytosol and nucleus. SRPK1 is the most studied family member, but little information is available concerning SRPK2 and SR proteins. SRPK1 preferentially phosphorylates serines within serine-arginine dipeptides, which is the most common pattern within the N-terminal portion of the RS domain (90, 91). One of the roles of SRPK1 is to phosphorylate SR proteins while they are located in the cytosol. Specifically, SRPK1 binds to a docking
motif within ASF/SF2 that directs its phosphorylation to ten serines within the N-terminal portion of the RS domain (91, 92). These phosphorylations enable ASF/SF2 to bind to transportin, enter the nucleus, and localize to speckles (91). As further evidence for the importance of SRPK1 in nuclear import, ASF/SF2 is unable to be transported from the cytosol into the nucleus in yeast lacking Sky1p, the yeast homologue of SRPK (77). SRPK1 can also contribute to the movement of SR proteins intra-nuclearly as SRPK1 over-expression causes dispersal of ASF/SF2 and SC35 from nuclear speckles (93). Although ASF/SF2 and SC35 are the most-studied substrates for SRPK1, it can also phosphorylate SRp55 and U2AF65 (90); however, a rigorous test of the action of SRPK1 upon all SR proteins is critically needed.

One mechanism in which the nuclear activity of SRPK1 is regulated is by its sequestration in the cytoplasm during certain phases of cell growth and in response to specific signals. In interphase cells, SRPK1 is mainly localized in the cytoplasm, but some is visible in the nucleus (94). A significant transition occurs prior to metaphase in

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Figure 4. Shuttling of SR Proteins. SR proteins shuttle between different cellular compartments in response to kinase and phosphatase activity. Transp SR = transportin SR, TAP = TAP exporter, PP = phosphatase. Adapted from Ngo et al., 2005.
which the majority of SRPK1 moves into the nucleus (94). A unique spacer sequence that is located between its two kinase domains functions to maintain SRPK1 in the cytoplasm. Deletion of the spacer sequence causes transport into the nucleus, resulting in phosphorylation of splicing factors (94-97). One mechanism for maintaining SRPK1 in the cytoplasm is its interaction with co-chaperone proteins. Upon cellular stress induced by osmotic shock, the co-chaperones dissociate, and SRPK1 enters the nucleus to phosphorylate SR proteins (98). Further research is needed, however, to determine whether SRPK1 translocation occurs in response to other signals and if the same regulatory proteins are involved. It is also important to understand how the activity of SRPK1 is regulated in the cytoplasm.

Clk/Sty kinase (cdc2-like kinase/serine/threonine/tyrosine kinase) also phosphorylates SR proteins. Humans express four isoforms of Clk/Sty, and they are members of the LAMMER family of protein kinases, named for the common LAMMER motif within the kinase domain (99). Clk/Sty contains an RS domain itself, it phosphorylates serine residues in SR proteins, it is located in the nucleus, and co-localizes to nuclear speckles with SR proteins. In general, Clk/Sty phosphorylates the same SR proteins that SRPK1 can and directly interacts with ASF/SF2, SRp20, SC35, SRp40, SRp55, and SRp75 (100, 101) but can phosphorylate other non-SR proteins (90). Clk/Sty tends to phosphorylate serines that are followed on the C-terminal side by arginines and prolines (90). Over-expression of Clk/Sty causes ASF/SF2 to move from the speckles to the nucleoplasm, creating a diffuse appearance (91, 100). Reciprocally, over-expression of a mutant Clk/Sty causes it and SR proteins to remain in speckles (100). The phosphorylation of SR proteins by Clk/Sty also enhances their interaction with other splicing factors. As an example, phosphorylation of ASF/SF2 via Clk/Sty enhances its interaction with U1 snRNP, inducing the splicing of a pre-mRNA in vitro (75). Clk/Sty can phosphorylate all 22 serine residues within the RS domain of ASF/SF2, while SRPK1 phosphorylation is restricted to ten serines within the N-terminal portion of the RS domain (92).

The regulation of Clk/Sty activity is complex and poorly understood. Autophosphorylation on tyrosine residues is one mechanism by which the activity of Clk/Sty activity is regulated (102). This regulation is quite complex as the combination
of autophosphorylated residues determines the SR proteins with which Clk/Sty can interact (103). Additionally, different patterns of autophosphorylation cause the kinase to phosphorylate different patterns of residues in the RS domain of ASF/SF2 (103). Clk/Sty is also phosphorylated by other kinases. A recent study indicates that, in response to insulin treatment, Akt2 phosphorylates Clk/Sty, which activates the kinase and induces the phosphorylation of several SR proteins (89). A better understanding of the regulation of Clk/Sty activity is critically needed. Questions to be addressed are, how autophosphorylation and phosphorylation by other kinases is coordinated, is there a compartmentalization factor regulating Clk/Sty activity, what circumstances control autophosphorylation, and does Clk/Sty phosphorylate all SR protein members?

Other kinases phosphorylate SR proteins besides SRPK1 and Clk/Sty. In vitro phosphorylation studies indicate that PKC and PKA phosphorylate ASF/SF2 (90), although no cellular consequence is known. Recently, it has been determined that insulin and other growth factors, through the action of Akt, induce phosphorylation of several SR proteins including SRp40, ASF/SF2, and 9G8, which has consequences upon the splicing of model pre-mRNAs (89, 104, 105). These studies were some of the first to find a link between extracellular signals, SR protein phosphorylation, and splicing.

**SR proteins and phosphatases**

Dephosphorylation of proteins in the spliceosome is also necessary for the splicing process. Initial in vitro splicing studies demonstrated that the activity of phosphatases upon snRNPs and ASF/SF2 is necessary for the first transesterification reaction of splicing (106, 107). Further dephosphorylation is required for the protein rearrangements that occur during conversion from the first to the second step of transesterification (107). These studies indicate that, in general, dephosphorylation of snRNPs and other proteins of the spliceosome is required for splicing catalysis and intron removal.

SR proteins are also direct targets of phosphatases. PP1 is an SR protein phosphatase that has been the most characterized. In addition to the effect of dephosphorylation upon transesterification steps, dephosphorylation of SR proteins also
influences the outcome of splicing (108-110). For example, an increase in ceramide in response to heat shock activates PP1 activity, which dephosphorylates SR proteins and impacts the splicing of several pre-mRNAs (109, 111), most likely through changes in protein-protein interactions. Additionally, hypophosphorylation appears to be a prerequisite for the export from the nucleus into the cytosol of SR proteins bound to spliced mRNA via interaction with the TAP exporter (112, 113). From there, phosphorylation via SRPK1 allows import back into the nucleus through their interaction with transportin-SR, and the cycle can begin again (114).

Although very little research has directly addressed what the differences are or if there are differences between alternative and constitutive splicing processes, phosphorylation status of SR proteins may be one distinguishing factor. Using well-defined splicing substrates and purified ASF/SF2, the Manley group determined that dephosphorylation is required for its activity in constitutive splicing but not upon a substrate that undergoes activated splicing, i.e., regulated alternative splicing (115). This study indicates that this differential requirement for phosphorylation upon two types of splicing substrates may be due to the ability of ASF/SF2 to interact with snRNPs. Specifically, more phosphorylation enhances the interaction of ASF/SF2 with the 70 kD subunit of U1 snRNP compared to mock phosphorylated ASF/SF2. Differences in phosphorylation had no effect upon the ability of ASF/SF2 to bind to U2AF35, however (115). This study provides the only direct comparison between the activity of SR proteins upon alternative and constitutive splicing substrates; thus, additional research is necessary to address this question and to determine if other differences exist between these two types of splicing modalities.

Significant research is still needed to better understand the consequences of phosphorylation and dephosphorylation upon SR protein action. One of the main obstacles to this area of research is the technical difficulty in detecting multiple phosphorylations within the highly repetitive RS domain; mass spectrometric analyses have been especially hindered. Of note, most of the research in this area has utilized ASF/SF2 as the prototypical SR protein. However, conclusions made about ASF/SF2 may be inapplicable to other SR proteins.
Other mechanisms of the regulation of SR protein activity

SR protein activity is regulated by several mechanisms besides covalent modifications of the RS domain, which includes changes in the amount of functional protein through transcriptional, pre-mRNA processing, and post-transcriptional mechanisms. A change the amount of the mature protein can be mediated through alternative splicing. Some SR proteins regulate their own expression, which is termed auto-regulation, and this occurs via alternative splicing that is coupled to NMD. As an example, exon 4 of SRp20 is an alternatively spliced exon and contains a premature stop codon. When SRp20 protein reaches a critical level, SRp20 binds to exon 4 of its own transcript and causes inclusion of exon 4. This leads to inclusion of the premature stop codon and degradation of the pre-mRNA by NMD (84, 116). ASF/SF2 antagonizes this series of events by binding to exon 4 and promoting exclusion of exon 4; and therefore, more SRp20 is translated (84). Inclusion of exon 4 has been observed in cells that are serum starved, and treatment with serum has been shown to reverse this effect (117). This mode of regulation with splicing coupled to NMD appears to be common for splicing factors (SRp40, SC35, SRp75, SRp30c, and Tra2β are also regulated similarly) and allows homeostatic regulation of SR protein abundance (116, 118, 119).

Another mechanism by which the expression of SR proteins is regulated is through proteosomal degradation. SRp55 protein is degraded in the proteosome when it becomes hyperphosphorylated by Clk/Sty overexpression. This is specific for SRp55 and not ASF/SF2 nor SC35, but the cellular consequences of this pathway are unknown (120).

Very little is known about the transcriptional control of SR protein expression. One of the only examples is transcriptional regulation of SRp20. Functional analysis of the promoter of SRp20 indicates that it is transcriptionally regulated by beta-catenin/TCF4 in colon cancer cell lines (117, 121). Overexpression of beta-catenin/TCF4 in colon cancer cells activated transcription of endogenous SRp20 mRNA as well as a luciferase reporter driven by the SRp20 promoter. The increase in accumulation of SRp20 mRNA and, subsequently, protein caused changes in alternative splicing of the CD44 pre-mRNA in these cells. In a related observation, an increase in
expression of SR proteins has been observed in cancer cells and tumors (122), which may be related to an increase in transcriptional activity of these SR proteins as is observed with SRp20, but this remains to be tested. Information regarding the transcriptional regulation of other SR proteins is lacking and may indicate that this is an unexplored area of research.

SR Proteins in other areas of gene expression

SR proteins are functionally linked to several other areas of gene expression besides pre-mRNA splicing, and the relationship between transcription and splicing has been an active area of research for many years. As splicing occurs while the pre-mRNA is being transcribed from the DNA template, it has been a widely held and supported concept that the CTD of Pol II is involved in recruitment of SR proteins to target pre-mRNAs. Several reports indicate that the CTD, when phosphorylated, recruits some members of the SR protein family to bind to it (123-126), and a proteomic analysis of the Pol II complex revealed that SR proteins and the U1 snRNP were bound to it (127). The involvement of the CTD in recruitment of SR proteins is controversial, however. Recent “splicing factor” ChIP (chromatin immunoprecipitation) experiments performed by the Neugebauer group indicate that there exists no direct link between the CTD and SR proteins. In these experiments, protein-DNA and protein-protein complexes were crosslinked within living cells, the SR proteins were immunoprecipitated, and quantitative PCR was performed to amplify distinct regions of the gene of interest, indicating where upon the pre-mRNA SR proteins were located. They show that when Pol II is paused at the transcription start site upon treatment with α-amantin, no SR proteins are “pre-loaded” upon the polymerase (71). Only after active transcription has begun are SR proteins associated with Pol II, and this only occurs through the RNA intermediate, not protein-protein interactions (71). The discrepancy between the data from the Neugebauer group and previous studies may be attributed to the rearrangement of protein interactions during immunoprecipitations. In vivo crosslinking followed by immunoprecipitation, performed by the Neugebauer group, is mostly resistant to these confounding effects due to the covalent bonds between the proteins and nucleic acids.
Although evidence indicates that SR proteins aren’t directed to splice sites via their binding to the CTD of Pol II, many properties of transcription, in general, influence and may regulate the splicing process. The speed of transcription can affect the outcome of alternative splicing (128, 129). A mutant Pol II with a slow elongation rate results in an increase in the inclusion of an alternative exon in the fibronectin EDI exon (130). Natural pause sites for Pol II also are important for the regulation of alternative splicing of immunoglobulin mu and α-tropomyosin (131, 132). The rate of transcription may influence the secondary structure of the pre-mRNA and therefore, have consequences for the ability of splicing factors to bind to target sequences (133) or the rate of appearance of competing cis-acting sequences can also be important for the proper ordering of alternative splicing (132). The rate of transcription can be affected by histone modifications, adding an additional layer of splicing regulation. Conversely, transcriptional activators, which enhance elongation rate, influence the alternative splice site selection as well as modulate constitutive splicing (134, 135). Different promoters driving expression of the fibronectin gene produce different alternative splicing events, indicating that promoter structure and/or transcription factors recruited to the promoter may be involved in splicing factor recruitment (136, 137). These data indicate that the splicing of a particular transcript is complex and is likely regulated by not only splicing elements and factors but aspects of its transcriptional regulatory elements. This level of complexity may make it nearly impossible to generalize the splicing regulatory process across all transcripts.

In a reciprocal manner, SR proteins can influence transcription. A recent research article indicates that SC35 depletion stalls Pol II elongation and transcription due to diminished recruitment of the kinase pTEFb and, consequently, reduced phosphorylation of serine-2 residues in the CTD (138). This observation may be unique to SC35, however, because this SR protein exhibits DNA-binding properties that are absent from other SR proteins.

SR proteins are involved in mRNA export out of the nucleus into the cytosol. All SR proteins shuttle from the nucleus to the cytoplasm via their attachment to spliced mRNA except SC35, which doesn’t appear to leave the nucleus (71). An active role in the export process has been demonstrated for SRp20, 9G8, and ASF/SF2, and these SR
proteins, upon their dephosphorylation within the spliceosome, enhance the export of an intronless mRNA by recruitment of the primary export protein, TAP (112, 139-141). Currently it is unknown which phosphatases dephosphorylate SR proteins during splicing catalysis prior to mRNA export and whether the effect upon transport is a function distinct from splicing or if it involves the recruitment of additional proteins.

Due to the physical link between SR proteins and cytosolic mRNA, it is not surprising that SR proteins may also modulate translation. ASF/SF2 is the only member of the SR protein family to be implicated in increasing cap-dependent translation efficiency. An initial study indicates that ASF/SF2 associates with translating ribosomes and the binding of ASF/SF2 to a cytoplasmic reporter mRNA harboring an ASF/SF2 binding site enhances translation of this mRNA and, recently, this has been extended to several other mRNAs (48, 142). This effect was specific to ASF/SF2, which shuttles, and not for SC35, which doesn’t shuttle. Interestingly, neither SRp20 nor 9G8 enhance cap-dependent translation of reporter mRNAs despite their ability to shuttle and enhance mRNA export (142), though recent evidence suggests that SRp20 is a mediator of cap-independent translation of a viral RNA (143). ASF/SF2 appears to enhance translation initiation by maintaining the translation factor 4E-BP in a hyperphosphorylated state that, resultantly, is unable to bind to eIF4E and inhibit translation (144). This occurs through the interaction of ASF/SF2 with mTOR kinase and/or the phosphatase PP2C (144). In another mechanism for the enhancement of cap-dependent translation, ASF/SF2 overexpression enhances the splicing and production of isoform 2b of MNK, which then phosphorylates eIF4E (122). All of these effects of SR proteins upon mRNA processing/localization may indicate that the nuclear history of an mRNA may determine its fate in the cytoplasm (48).

**Alternative splicing regulation**

A main goal in the field of splicing is to understand how external stimuli affect the splicing of pre-mRNAs, which is generally termed regulated splicing. In general, regulated splicing occurs through the interaction and competition between enhancer and silencer elements and proteins. Perhaps, the best-characterized model of regulated
splicing in higher eukaryotes is the CD45 gene in T-cells. CD45 is a transmembrane tyrosine phosphatase that undergoes alternative splicing in response to T-cell activation. Specifically, T-cell activation causes exclusion of exons 3, 4, and 5 to create an inactive phosphatase that is necessary for the maintenance of T-cell homeostasis (145). An ESS within exon 4 is one important cis regulatory element responsible for activation-induced exon skipping, and hnRNP LL is the trans-acting mediator (146, 147). The expression of hnRNP LL is up-regulated in response to T-cell activation (146), but the signaling pathways that produce this response are still unknown. This model exemplifies how activating signals can modulate the splicing of a particular pre-mRNA through a change in the expression of a splicing factor, which produces important consequences for cellular homeostasis. Of particular note, recent evidence indicates that the increase in expression of hnRNP LL affects the splicing of a “program” of target genes that are responsible for T-cell activation (147). This study demonstrates for the first time a “global” splicing regulatory system dependent upon a single splicing factor.

A seminal paper in understanding how alternative splicing is regulated by growth factors was published in 2005 by the Srebrow group. They showed that treatment of cells with growth factors caused an increase in phosphorylation within the RS domain of ASF/SF2 and 9G8, and Akt was the direct mediator of these phosphorylation events (104). Additionally, the increase in phosphorylation of these two SR proteins coincided with an increase in inclusion of the EDA exon within a fibronectin mini-gene reporter. Furthermore, growth factors and Akt activation enhanced the translation efficiency of a reporter mRNA. This study was groundbreaking in the field of splicing and greatly facilitated further study into the impact of other growth factors, such as insulin, upon splicing.

The impact of insulin upon splicing processes is a developing field of study. Using exon–array analysis in drosophila cells, the Valcarcel group estimates that greater than 150 genes undergo alternative splicing upon insulin treatment, with genes involved in amino acid and carbohydrate metabolism being the second most affected family (148). Forty percent of the genes that are affected at the level of splicing are also regulated at the transcriptional level (148). In higher eukaryotes, specific pre-mRNA transcripts that
undergo alternative splicing in response to insulin and other growth factors include PKCβII, the insulin receptor, and fibronectin EIIIB (149-151).

The signaling mechanism involved in the effect of insulin upon PKCβII splicing is a prime example of how kinases affect the splicing process. Work in the Cooper lab has determined that insulin treatment in muscle cells activates Akt2, which then directly phosphorylates SRp40, activating its splicing function upon PKC pre-mRNA (105). The binding of SRp40 to PKC pre-mRNA induces exon inclusion and production of PKCβII splice product, which enhances glucose uptake. As another layer of regulation, Akt2 also phosphorylates Clk/Sty in response to insulin; this activates Clk/Sty to phosphorylate SRp75, SRp55, and SC35 (89). These studies indicate that insulin has the potential to affect the splicing of many target transcripts in different tissues in higher eukaryotes as it does in drosophila. High-throughput screening technologies such as exon-junction microarrays and high throughput sequencing will enable global screening of splicing events that are induced by external stimuli.

As the nutrient-responsive hormone, insulin, is a modulator of splicing, it follows that nutrients per se are also regulators of alternative splicing. One of the only examples of a global view of alternative splicing in response to nutrients is in *Saccharomyces cerevisiae*. Exon junction array analysis results indicate that amino acid starvation treatment reduces the splicing efficiency of several pre-mRNAs, and gene annotation identified pre-mRNAs coding ribosomal proteins were most severely affected (152). Additionally, ethanol treatment of the cells altered the splicing of a non-overlapping, discrete family of pre-mRNAs.

Bioactive lipids are important regulators of many aspects of gene expression, but their role in splicing is just beginning to be evaluated. Ceramide is one example of a bioactive lipid that regulates splicing. An increase in ceramide activates PP1 activity, which reduces SR protein phosphorylation in cells (111); this has the potential to cause changes in splicing of many pre-mRNAs. Ceramide directly regulates the splicing of two pre-mRNAs including caspase-9 and Bcl-x between their pro-apoptotic and anti-apoptotic isoforms by the differential recruitment of splicing factors (153-155). Ceramide levels are elevated by several fatty acids including arachidonic acid and linolenic acid, which increase the metabolism of sphingomyelin to ceramide (156). Fatty
acids also inhibit the splicing of the lipogenic enzyme, glucose-6-phosphate dehydrogenase (G6PD), which will be discussed in greater detail later in this chapter.

**Splicing and disease**

Aberrant splicing can result from mutations in *cis* elements or dysregulation of *trans* elements, which can lead to disease (83, 157, 158). It is estimated that greater than 50% of the point mutations that cause disease affect splicing (83). One of the most studied cases of a point mutation disrupting splicing and resulting in disease is within the SMN2 gene (survival of motor neuron 2), a protein that is necessary for snRNP synthesis. A single point mutation within exon 7 leads to exon skipping creating an unstable protein that results in the disease called spinal muscular atrophy. The point mutation is thought to disrupt an ESE to which ASF/SF2 binds and/or create an ESS to which hnRNP A1 binds (159, 160). Research efforts are currently underway to develop gene therapy to correct this splicing defect and have produced some promising results. This example illustrates how the delicate balance between positive and negative regulators of splicing can go awry.

Elevated levels of splicing factor proteins are also correlated with disease. There is a strong correlation between increased expression of SR proteins and several different types of cancer. Recently, the Krainer group directly demonstrated the pro-oncogenic properties of ASF/SF2 (122). Overexpression of ASF/SF2 is tumorigenic in animal models, and this protein appears to transform cells via changing the splicing patterns to create pro-oncogenic and anti-apoptotic versions of proteins and also through non-splicing mechanism involving mTOR (122, 161). As these examples illustrate, the delicate balance between the amounts of *trans*-acting splicing factors and the fidelity of the sequences of *cis* regulatory elements must be maintained for normal cellular function.

**Summary**

Splicing is a complex process, and all of the regulatory factors impinging upon it are just beginning to be understood. As discussed above, constitutive and alternative
splicing are regulated by the interaction between cis and trans elements. SR proteins are a major family of splicing regulatory proteins that generally enhance the formation of the spliceosome through several mechanisms. Their activity is regulated through post-translational modifications, sub-cellular localization, and changes in expression profiles. They have been shown to mediate the effect of extracellular signals upon the splicing of pre-mRNA targets in a few examples, but this area is poorly understood. G6PD is a model mRNA that undergoes differential splicing in response to starvation and refeeding in whole animal. This model has the potential to produce a better understanding of the impact of nutrients upon the activity of SR proteins and target pre-mRNAs.

The regulation of G6PD splicing by nutrients

G6PD oxidizes glucose-6-phosphate to 6-phosphogluconolactone in the first rate-determining step of the pentose phosphate pathway, which is also known as the hexose monophosphate shunt and the phosphogluconate pathway. This reaction reduces NADP⁺ to NADPH + H⁺ which then provides reducing power for biosynthetic reactions such as fatty acid synthesis, cholesterol synthesis, and amino acid synthesis (162). The NADPH + H⁺ is also necessary for maintaining glutathione in its reduced form for protection against reactive oxygen species. The ribose-5-phosphate produced by the pentose phosphate pathway is utilized in nucleotide biosynthesis.

As glucose-6-phosphate is a substrate for both glycolysis and the pentose phosphate pathway, cellular needs determine where the sugar is directed. This regulation occurs through the change in activities of phosphofructokinase, one of the main regulatory enzymes of the glycolytic pathway, and G6PD. Phosphofructokinase activity is inhibited when the ATP/AMP ratio is high and by citrate, which slows the flux through the glycolytic pathway. High levels of NADPH and fatty acids inhibit G6PD activity, and, thus, the flux through the pentose phosphate pathway decreases. A change in the expression of G6PD is the mechanism by which its activity is regulated in the metabolic tissues of the liver and adipose tissue, and its expression is regulated by nutrients and hormones.
The process of fatty acid biosynthesis involves conversion of excess dietary carbohydrate to the fatty acid palmitate. The carbon required for fatty acid biosynthesis is derived from mitochondrial citrate from the TCA cycle, which crosses the inner mitochondrial membrane to the cytosol. There, ATP-citrate lyase converts the citrate to acetyl-CoA and oxaloacetate. The acetyl-CoA proceeds to fatty acid synthesis, while the oxaloacetate returns to the mitochondria either as malate or pyruvate through the activity of the citrate-malate-pyruvate shuttle in the cytosol. Malic enzyme, which converts malate to pyruvate in the citrate-malate-pyruvate shuttle, also provides about 50% of the NADPH + H⁺ required for fatty acid biosynthesis, and G6PD and 6-phosphogluconate dehydrogenase, the second enzyme of the pentose phosphate pathway, provide the other half of the reducing equivalents (162). The first committed step of fatty acid biosynthesis is performed by acetyl-CoA carboxylase, carboxylating acetyl-CoA to malonyl-CoA. All of the subsequent steps of palmitate synthesis occur through the action of fatty acid synthase, which is a dimer of identical 250 kD proteins containing multiple enzyme functions (Figure 5). The palmitate that is produced can be further elongated and desaturated to produce other types of fatty acids. Desaturation is performed by steroyl-CoA desaturase. Fatty acids are esterified to glycerol, forming triacylglycerol and can be transported from the liver to adipose tissue within VLDL.

The family of lipogenic enzymes consists of the following members: G6PD, malic enzyme, ATP-citrate lyase, acetyl-CoA carboxylase, and fatty acid synthase. The activity of these enzymes is regulated coordinately in liver and adipose tissue so that activity is low when the energy status and substrate availability is low, such as during a period of fasting. Alternatively, when nutrients exceed immediate energy needs, such as after a high carbohydrate meal, activity of these enzymes is enhanced to convert this energy to stored energy in the form of triacylglycerol in adipose tissue. The end-product of fatty acid synthesis, fatty acids, also inhibits lipogenic flux. The target of this inhibition is acetyl-CoA carboxylase, which can be regulated with covalent modification, in which phosphorylation inhibits its activity, providing immediate reduction in fatty acid synthesis. This phosphorylation is performed by AMP-activated kinase that is triggered to phosphorylate acetyl-CoA carboxylase when the AMP/ATP ratio is high. The
activities of most of the other lipogenic enzymes are regulated by changes in expression, with transcriptional regulation by sterol-response element binding protein being the most common mechanism. Transcriptional changes are regulated by hormonal cues from insulin and glucagon as well as dietary compounds such as polyunsaturated fatty acids and glucose.

G6PD expression, however, is regulated at a post-transcriptional step (163). When mice are fasted for 12 hr and then refed a diet that is high in carbohydrate and low in fat, hepatic G6PD expression is induced by 12-fold or greater compared to fasted mice (164, 165). There is a similar response in primary rat hepatocytes that are treated with insulin compared to glucose alone (166). On the other hand, G6PD expression is 60% lower in the livers of mice that are fed a diet containing 6% polyunsaturated fat compared to a low fat (1%), high carbohydrate diet (164). Mirroring this effect in primary culture, arachidonic acid attenuates the insulin-induced increase in G6PD expression in rat hepatocytes by 40-60% (167).
Despite large changes in mRNA abundance, the transcription rate of the G6PD gene does not differ between dietary treatments (165, 167, 168). In addition, other points of RNA processing, including polyadenylation rate and length of the poly (A) tail, are not altered in refed or starved mice (168). The regulatory point affected by polyunsaturated fatty acids is pre-mRNA splicing. Arachidonic acid treatment reduces the efficiency of splicing by causing retention of intron 11 (167), which reduces the amount of mature G6PD mRNA that can be exported to the cytosol for translation. At the molecular level, recent work in our lab indicates that arachidonic acid treatment inhibits expression of G6PD mRNA by interfering with the action of insulin. Specifically, arachidonic acid treatment results in the activation of p38 MAP kinase, which causes IRS-1 to become phosphorylated at serine 307, thereby reducing activation of the phosphoinositide 3-kinase pathway (169).

We have identified an ESS that governs G6PD splicing repression during treatment with arachidonic acid. A reporter system was used to define the G6PD regulatory sequence in primary rat hepatocytes. Several reporters were constructed that contained different regions of the G6PD driven by the CMV promoter. These reporters were transfected into primary rat hepatocytes and the cells were treated with insulin or insulin plus arachidonic acid. The inhibition of expression of each reporter in response to arachidonic acid was evaluated with RNase protection assays. These experiments determined that exon 12 was necessary for the inhibitory effect of arachidonic acid as reporters containing exon 12 were inhibited similarly to endogenous G6PD mRNA (167, 170). Thus, the reporter system was an accurate model of the endogenous G6PD mRNA. To further define the regulatory sequence within exon 12, nine constructs were synthesized that contained exons 7-13, their intervening introns, and a span of 10-nt block mutations (AAGCATGCAA) within exon 12. Upon expression in primary hepatocytes, three constructs with mutations spanning nucleotides 43 to 72 of exon 12 lacked appreciable inhibition of splicing in the presence of arachidonic acid compared to insulin alone (170)(~10% only). Since this region is important for inhibition of splicing, we suggest that it contains an ESS.

*In vitro* splicing assays provided additional evidence that exon 12 contains an ESS. RNA substrates designed with and without exon 12 were *in vitro*-transcribed in the
presence of $^{32}$P-labeled CTP to label the RNA and incubated with HeLa nuclear extract, which provides the necessary splicing proteins. Substrates lacking exon 12, such as exon 8-intron 8-exon 9, exon 10-intron 10-exon 11, and exon 10-intron 11-exon 11 exhibited splicing (60% to 70% spliced). However, exon 11-intron 11-exon 12, exon 10-intron 10-exon 12, and exon 8-intron 8-exon 12 were not spliced (less than 5% spliced) (170).

Next, spliceosome assembly assays were performed to determine at what step the assembly of the spliceosome was being inhibited. These experiments are identical to in vitro splicing assays except that the reactions are stopped at several different time points with the addition of heparin. The progress of spliceosome assembly through time is visualized by the characteristic sizes of protein-RNA complexes on polyacrylamide gels. Specifically, the H and E complexes exhibit the fastest gel mobility, followed by the A complex, and finally the largest complexes are the B and C complexes, which migrate together and form just prior to splicing catalysis. These experiments indicated that the exon 11-intron 11-exon 12 substrate, compared to positive controls, exhibited reduced formation of the A complex and completely lacked formation of the B and C complexes. The A complex forms when U2 snRNP binds to the intron; thus, this indicates that the ESS represses splicing of intron 11 by preventing the recruitment of U2 snRNP (170).

In addition to this region containing an ESS, experiments indicated that it also contains an ESE. A deletion of the regulatory element in exon 12 from the exon 11-intron 11-exon 12 substrate did not confer splicing activity upon this substrate in an in vitro splicing assay (170). This indicated that deletion of the regulatory element not only removed an ESS but also an ESE. As juxtaposed ESE and ESS elements are a common regulatory mechanism in splicing, and since our data supported this concept, we hypothesized that the regulatory element contains both an ESS and an ESE that regulate differential splicing in response to nutrients.

In order for a cis-acting ESS to produce an effect on splicing, trans-acting proteins must bind to the sequence and influence the formation of the spliceosome. We have identified putative splicing repressor proteins that bind to the regulatory region of exon 12 through UV crosslinking analysis and RNA affinity techniques. RNA oligonucleotides corresponding to 15 nucleotide spans of exon 12 were incubated with starved and refed mouse liver nuclear extract, the oligonucleotides and nuclear proteins
were crosslinked, and the complexes were treated with RNase to remove unprotected bases. The proteins were run on a SDS-PAGE gel and visualized by phosphorimaging to detect their sizes. The oligonucleotide spanning nucleotides 65-79 of exon 12 consistently was bound by proteins of 60 and 37 kDa molecular weight (171), and similar results were found with RNA affinity experiments. Through liquid chromatography-tandem mass spectrometry, the 60 kDa band was identified as hnRNP K and L, and the 37 kDa band was identified as hnRNP A2/B1 (170, 171). HnRNP A2 and B1 are two closely related proteins that only differ by an additional 12 amino acid residues in B1. A peptide was sequenced in the tandem mass spectrometry experiment that corresponded to this 12 amino acid region, which indicated that B1 was present; however, we could not exclude the possibility that A2 was also present as the two proteins are otherwise identical. Thus, these hnRNPs bind specifically to the 50-79 nucleotide region that overlaps the splicing regulatory element region, nucleotides 43-72 of exon 12, and we hypothesize that they are necessary for the repression of splicing of G6PD.

Evidence for the role of these hnRNPs in the repression in G6PD splicing was observed with liver nuclear extract from starved and refed mice. Western analysis was performed after the nuclear proteins were purified with the oligonucleotide corresponding to the splicing regulatory element. The western analysis confirmed that hnRNP K, L, and A2/B1 bound to the splicing regulatory element. We additionally observed a 60-70 % reduction in binding of these proteins to the 50-79 oligonucleotide in nuclear extracts from the livers of refed animals, in which G6PD splicing was enhanced, compared to the extracts from the livers of starved animals, in which splicing was inhibited (171). Thus our lab has demonstrated that hnRNP K, L, and A2/B1 bind in greater abundance to the splicing regulatory element of exon 12 in the starved nutritional state.

**Hypothesis**

Understanding the activity of the splicing machinery is affected by external stimuli is a crucial area of splicing research. As discussed above, very few examples exist in the literature that directly address this question. G6PD is an excellent model with which to study the how nutrients regulate the splicing process differentially. We have
identified a splicing regulatory element within exon 12 of the G6PD transcript, which exhibits ESS and ESE characteristics. Since we observe an increase in splicing efficiency in response to insulin treatment and refeeding and reduced splicing efficiency of G6PD in response to starvation and polyunsaturated fatty acids, we hypothesize that this differential splicing is due to competition between closely juxtaposed ESS and ESE elements within the splicing regulatory element. As previously characterized inhibitory splicing proteins bind to the regulatory element during starvation when splicing is inhibited, we hypothesize that SR proteins bind to the element during refeeding when splicing is enhanced. Therefore, we hypothesize that SR proteins are more active during refeeding than during starvation and that this change in activity is due to changes in phosphorylation of the RS domain of these SR proteins. The data presented herein support our hypothesis that nutrients and insulin affect the activity of SR proteins upon the model pre-mRNA, G6PD, and this change in activity most likely occurs through changes in phosphorylation of SR proteins. Additionally, we provide evidence that SRp20 binds to the regulatory element of G6PD and enhances splicing of this transcript. These data are novel and have implications for the splicing of other transcripts in response to dietary changes. This knowledge is important for understanding the complex molecular mechanisms of the nutritional regulation of gene expression and diseases associated with excess nutrients such as obesity and Type II diabetes.
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CHAPTER 2. SUBMITTED TO MOLECULAR ENDOCRINOLOGY

SRp20 is involved in enhanced mRNA splicing in response to nutrients and hormones in liver¹.

Callee M. Walsh, Alison B. Kohan, Wioletta Szeszel-Fedorowicz, Travis J. Cyphert, and Lisa M. Salati

Department of Biochemistry, West Virginia University, Morgantown, WV

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Corresponding author: Dr. Lisa Salati; Department of Biochemistry, One Medical Center Drive, PO Box 9142 Health Sciences Center, Morgantown, WV 26506; Phone: (304) 293-7759; Fax: (304) 293-6846; e-mail: Lsalati@hsc.wvu.edu

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ESS (exonic splicing silencer), glucose-6-phosphate dehydrogenase (G6PD),
phosphoenolpyruvate carboxykinase (PEPCK), RNA polymerase II (Pol II), arginine-serine rich domain (RS domain), serine-arginine rich protein (SR protein)

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Reprint requests to:
Lisa M. Salati
Department of Biochemistry
One Medical Center Drive
PO Box 9142 Health Sciences Center
Morgantown, WV 26506

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Abstract

Dietary status and individual nutrients and hormones regulate alternative splicing of eukaryotic mRNAs. We have established a model for this type of regulation by analyzing the abundance of glucose-6-phosphate dehydrogenase (G6PD) mRNA in liver in which its splicing is enhanced during refeeding/insulin treatment and splicing is attenuated during starvation/treatment with polyunsaturated fatty acids. Differential splicing of G6PD mRNA involves a regulatory element in exon 12, which contains a putative exonic splicing enhancer sequence. The primary goal of the current studies was to determine whether nutritional status affects the activity of SR proteins and to establish changes in SR protein activity as a mechanism for nutritional regulation of mRNA splicing. Consistent with the G6PD regulatory element containing an exonic splicing enhancer, SRp20 and SRp75 bound to the regulatory element, and binding was enhanced by refeeding, corresponding to an increase in splicing. Refeeding also enhanced the binding of SR proteins to the G6PD transcript in vivo as identified by chromatin immunoprecipitation. In hepatocytes, insulin stimulated phosphorylation of several SR proteins, while addition of arachidonic acid inhibited this effect. In vitro splicing assays and siRNA-mediated knock-down of SRp20 demonstrated that SRp20 enhances splicing of G6PD. Together, these data establish that nutritional regulation of SRp20 activity is involved in the differential splicing of the G6PD transcript in response to nutrients; in addition, nutritional regulation of other SR proteins suggests that this regulatory mechanism is significant for widespread changes in mRNA splicing.
Introduction

Greater than 90% of human transcripts undergo alternative splicing which can alter many functional aspects of the resulting proteins, including, but not limited to, the amount expressed, the binding properties of the protein, and the presence or absence of specific domains within the protein (1, 2). Changes in splicing patterns are involved in development and maintaining the phenotype of somatic cells (2). Splicing is regulated by *cis*-acting elements and *trans*-acting factors; therefore, mutations in these elements or changes in the activity of *trans*-acting proteins will alter cellular function and potentially lead to disease (3). In order to recognize the contribution of splicing to cellular function, it is necessary to understand how splicing is regulated in different cell types by extracellular signals.

Serine-arginine rich (SR) proteins are a family of *trans*-acting splicing factors that activate splicing (4). The canonical SR protein family members include SRp75, SRp55, SRp40, ASF/SF2, SC35, 9G8, and SRp20. These proteins enhance the splicing process by binding to exonic splicing enhancers (ESEs) to recruit U1 snRNP, U2AF<sup>35</sup>, and U2 snRNP to intronic splice sites and/or by inhibiting the binding of negative regulators of splicing (5, 6). SR proteins play a critical role in the splicing of alternatively included exons and are also essential for constitutive exon splicing when the surrounding intron has weak splice sites (4). The structural characteristic of SR proteins is one or two RNA recognition motifs and an arginine-serine rich (RS) domain. The RS domain is phosphorylated on serines, which enhances the interaction of SR proteins with other splicing factors (4). Phosphorylation also regulates intra-cellular and intra-nuclear localization of SR proteins, which controls their access to target transcripts (7). As the RS domain contains many serines that can be phosphorylated and are substrates of several kinases, SR proteins are central players that mediate the impact of extracellular signals upon the splicing process (8-10). The involvement of hormones and other extracellular signals in regulating splicing factor activity is a poorly understood area.

The overriding question of our research is to understand how nutrients affect the splicing process, and we utilize a model gene, glucose-6-phosphate dehydrogenase (G6PD) to address this question. G6PD is an enzyme that is integral to the process of *de*
novo lipogenesis in liver and adipose tissue by its generation of reducing equivalents in the form of NADPH + H⁺ (11). Its expression is regulated in these tissues by nutrients, and this regulation occurs by changes in the rate of splicing of the G6PD pre-mRNA (12). In this regard, in rodents, starvation or consumption of a diet containing polyunsaturated fatty acids reduces the efficiency of intron removal from the primary transcript (13), subsequently leading to reduced expression of the enzyme, and, therefore, fewer reducing equivalents are available for lipogenesis. This is a form of regulated alternative splicing called intron retention, and it shares cis-acting elements and trans-acting factors with other forms of alternative splicing. G6PD pre-mRNA containing retained introns adjacent to exon 12 accumulates in the nucleus when splicing is inhibited. Feeding a high carbohydrate, low fat diet to rodents after a short-term fast, which is termed refeeding, induces efficient splicing, greater expression of the enzyme, and an increase in lipogenic capacity compared to animals that are fasted (12). These in vivo feeding experiments are recapitulated in primary rat hepatocytes in culture in which treatment with insulin induces the accumulation of spliced G6PD mRNA, and the polyunsaturated fatty acid, arachidonic acid, attenuates mRNA splicing (13-15). A splicing regulatory element in exon 12 of the G6PD transcript mediates the effect of nutrients upon the splicing of this pre-mRNA (15).

In the current set of experiments we sought to determine whether the activity of SR proteins changes in response to nutrients and also identify candidate SR proteins that mediate the increase in splicing of G6PD that occurs in response to refeeding and insulin treatment. Using G6PD as a prototype, we determined that refeeding increased the binding of SR proteins to the splicing regulatory element both in vitro and in vivo. Furthermore, insulin increased the nuclear content of phosphorylated SR proteins, while arachidonic acid reduced this effect. Within the family of SR proteins, SRp20 was specifically involved in the regulation of G6PD mRNA splicing. Overall, these findings provide not only a greater understanding of stimuli and signaling events that affect the splicing process but further define molecular mechanisms of metabolic regulation.

Results
Previously, we have determined that an RNA regulatory element necessary for differential splicing is located within nucleotides 43-72 of exon 12 of G6PD mRNA, and this regulatory element contains an exonic splicing silencer (15). The ESS in this sequence is necessary for the inhibitory effect of arachidonic acid upon splicing in primary rat hepatocytes, it suppresses splicing of chimeric splicing substrates, and it binds three members of the hnRNP family (K, L, A2/B1) coincident with splicing attenuation (15, 16). Deletion of the 43-72 nucleotide regulatory element fails to restore splicing in *in vitro* assays, suggesting the presence of an ESE.

**Refeeding increases the binding of SR proteins to the splicing regulatory element.**

The goal of the current series of experiments was to determine if SR protein binding was relevant to nutritional stimulation of splicing and to determine which SR proteins are involved in the splicing of G6PD in response to nutritional cues. We hypothesized that if SR proteins mediate the increase in splicing that is observed during refeeding and insulin treatment, then their binding to the regulatory element would be enhanced under these dietary treatments. An RNA affinity assay was performed to first identify which SR proteins bound to the element and, second, to determine if the binding was regulated by nutritional state (Fig. 1). Nuclear extracts were prepared from the livers of rats that had been starved for 18 h or starved and then refed a high carbohydrate diet for 16 h. Refeeding increases the accumulation of G6PD mRNA an average of 8-fold above that observed in the livers of starved animals, and this was confirmed in these livers (12, 17 and data not shown). In the RNA affinity assay, RNA oligonucleotides corresponding to the splicing regulatory element (nucleotides 43-72 of G6PD exon 12) were attached to agarose beads, and used to affinity purify RNA binding proteins from the fasted and refed nuclear extracts. An RNA oligonucleotide corresponding to a region of exon 12 outside of the regulatory element (control region; nucleotides 79-93) was used to control for non-specific binding of proteins to RNA, and the beads alone were included to detect any background binding contributed by the beads. Binding proteins were eluted with a step-wise gradient of buffers containing an increasing amount of KCl, and SR proteins were detected using western analysis (Fig. 1). To screen for the binding of multiple SR proteins in a single blot, the SR protein antibody 16H3 was used, which
detects both the phosphorylated and non-phosphorylated forms of SRp75, SRp55, SRp40, and SRp20. As shown in Fig.1A, the low salt wash eluted SRp40, SRp55, and SRp75 from the splicing regulatory element, the control region, and beads in both dietary treatments, but only SRp75 maintained a persistently strong interaction with the regulatory element RNA as indicated by its elution in the higher salt buffers. SRp20 was undetectable in this blot due to its low abundance in liver compared to the other three SR proteins (Fig. 1C). To address this, a monoclonal antibody that detects SRp20 was used. Similar to SRp75, SRp20 was eluted with the lowest salt wash from all of the affinity columns and from both dietary groups (Fig. 1B). SRp20 remained bound to the regulatory element and was eluted at higher salt concentrations, but was not detected bound to the control RNA region or the beads alone. The persistent interactions of SRp20 and SRp75 with the regulatory element indicate a specific interaction between these *trans*-acting splicing regulators and the *cis*-acting RNA element from exon 12 of G6PD mRNA. Although SRp40 and SRp55 are also present in nuclear extract (Fig. 1C), they do not bind to the splicing regulatory element (Fig. 1A).

Notably, nutritional status of the animal regulated the binding of these SR proteins. Refeeding enhanced the binding of SRp20 to the RNA element by 3-fold. It also caused a small, but reproducible increase in the binding of SRp75 to the element (20%). The increase in binding upon refeeding was evident in spite of equal amounts of these two SR proteins in the nuclear extracts from both fasted and refed livers (Fig. 1C). The coincident changes in G6PD mRNA accumulation and SR protein binding in refed rat livers is consistent with our hypothesis that SR proteins enhance the splicing of G6PD mRNA in response to nutritional status. Thus, SRp20 and SRp75 are candidate SR proteins involved in this regulation.

If the interaction between SR proteins and the splicing regulatory element are important for the nutritional regulation of splicing, then these interactions should occur in intact cells. To determine if these interactions occurred within intact liver cells and during starvation and refeeding, we used the chromatin immunoprecipitation (ChIP) assay. ChIP is commonly used for detecting DNA-binding proteins bound to specific nucleic acid sequences within the chromatin (18); however, ChIP is becoming a standard technique for detection of RNA-protein interactions during transcription of the nascent
mRNA (19-21). This technique detects the binding of splicing factors to target pre-mRNA sequences via their close proximity to chromatin during the transcription and processing of mRNA (22). The co-transcriptional occurrence of splicing (23) and the close proximity of splicing factors and pre-mRNA to the chromatin (24) enable cross-linking between RNA-bound splicing factors and the adjacent chromatin. After cross-linking, the protein of interest is immunoprecipitated and the DNA sequences corresponding to the RNA region of interest are amplified by real-time PCR, which is used as a measure of the protein occupancy on the pre-mRNA.

ChIP was performed in livers from either starved or starved and then refed mice to detect the occupancy of phosphorylated SR proteins on G6PD pre-mRNA. The monoclonal antibody 1H4 was used that detects phosphorylated RS domains, and, therefore, recognizes all members of the SR protein family. Available antibodies for specific SR proteins do not perform well in ChIP assays, and, thus, this strategy is not used (21). The 1H4 antibody also cross-reacts with the phosphorylated C-terminal domain of RNA polymerase II (Pol II; 25). To correct for this, ChIP was also performed with an antibody against Pol II. Because the ChIP technique detects interactions between the RNA binding protein and nearby DNA, primers were designed to amplify a region encompassing the regulatory element and sequences upstream in the exon. We hypothesized that refeeding, which induces the splicing of G6PD, would increase the binding of phosphorylated SR proteins to the regulatory element of G6PD. As shown in Fig. 2, refeeding significantly induced the binding of phosphorylated SR proteins to the splicing regulatory element by 1.8-fold above that observed during fasting. Dietary treatment did not alter the occupancy of Pol II across this region of exon 12 indicating that a true increase in SR protein binding was occurring (Fig. 2). The lack of change in Pol II occupancy on the G6PD chromatin is consistent with the absence of transcriptional regulation of this gene (17). Next, we measured the occupancy of SR proteins on exon 2 of phosphoenolpyruvate carboxykinase (PEPCK) pre-mRNA, which is transcriptionally regulated and induced during starvation (26). Fasting induced the binding of phosphorylated SR proteins to PEPCK pre-mRNA by 2.7-fold over refeeding; however, starvation also increased the binding of Pol II to the PEPCK gene, consistent with the increase in transcriptional activity of this gene (26). Thus, the enhanced binding of SR
proteins to the G6PD regulatory element is not due to a generalized effect of refeeding upon the binding of SR proteins to RNA elements. Additionally, hnRNP M immunoprecipitation was performed as a negative control because previous experiments have determined that this RNA binding protein fails to bind to the splicing regulatory element in vitro (15). Binding of hnRNP M to the regulatory element was low, and this binding was not altered by the nutritional status of the animal. The ChIP assay, thus, extended the RNA affinity assay data and demonstrated that, in liver, refeeding induces the binding of SR proteins to the RNA element of G6PD. These ChIP data are quite novel as they are the first to demonstrate that SR proteins bind to target pre-mRNAs differentially in response to metabolic signals in the whole animal.

**SiRNA depletion of SRp20 decreases G6PD mRNA accumulation.**

Binding of SR proteins to the RNA element is consistent with the element being an ESE (27). The demonstration of regulated binding in intact tissues suggests a functional enhancer activity for the SR proteins. To address the function of SRp20 and SRp75 in regulating splicing of G6PD mRNA in cells, we used a loss-of-function approach. siRNA-mediated knock-down of SRp20, SRp75 and SRp55 was performed, and the effect upon expression of a G6PD splicing reporter and endogenous G6PD was evaluated. SRp55 knock-down was included as a negative control because this protein did not bind to the G6PD regulatory element (Fig. 1A). HeLa cells were used because they are easy to transfect, and the pattern of SR protein binding to the G6PD regulatory element is similar to liver (data not shown).

siRNAs targeting SRp20, SRp55, and SRp75 each faithfully depleted its respective protein and had little effect upon non-target SR proteins (Fig 3A). SRp20, SRp55, and SRp75 were reduced by 87%, 93%, and 94%, respectively, compared to the non-targeting siRNA (NT). Knock-down of SRp20 resulted in a small but reproducible decrease in SRp75, SRp40 and SRp30a/b, but this decrease was much less than the decrease in SRp20 itself. The expression of SR proteins involves not only auto-regulation of their own splicing but also regulation by other family members (28, 29); thus, some decrease is not unexpected. This western analysis was performed with
mAb104, which detects phosphorylated SR proteins (Fig. 3A, top panels), but antibodies detecting total amounts of these SR proteins showed similar results (data not shown).

The splicing reporter used in these experiments, pβ-gal ex12-ex13 (Fig. 3B), contains exon 12, intron 12, exon 13, and the 3' UTR of G6PD ligated to the β-galactosidase gene. This design allowed us to evaluate the role of these SR proteins upon splicing regulated by the element in exon 12 with minimal interference by ESEs in other exons of the G6PD pre-mRNA. Additionally, expression of this reporter is regulated in a manner similar to endogenous G6PD in primary rat hepatocytes treated with insulin and arachidonic acid (15). Reduction in SRp20 protein expression inhibited splicing of the reporter mRNA by 44% in comparison to cells transfected with NT siRNA (Fig. 3C, upper panels). In contrast, loss of SRp75 expression did not inhibit expression of the reporter mRNA and, in fact, enhanced expression 1.9-fold compared to NT siRNA. Knock-down of SRp55, which does not bind to the regulatory element, also enhanced expression (1.4-fold). An increase, rather than a decrease or no effect, in reporter expression was unexpected and may reflect peculiarities of the reporter RNA sequence.

As further investigation of the role of SRp20 in splicing of G6PD, we evaluated the effect of the SR protein knock-down upon the expression of the endogenous G6PD transcript. The interaction of splicing regulatory sequences and their associated binding proteins in the regulation of basal G6PD splicing is conserved in HeLa cells. Additionally, the expression of G6PD in these cells is quite robust, which suggests that “splicing activation” is the default. SRp20 protein depletion resulted in a 30% reduction in expression of endogenous G6PD (Fig. 3C, middle panels). This decrease in endogenous RNA splicing was remarkable as some cells most likely were not transfected with siRNA, and the 13 exons of the G6PD transcript undoubtedly contain multiple splicing regulatory elements that interact with other SR proteins. SRp55 also significantly reduced the expression of this transcript by 26%, but SRp75 had no significant effect. The effect of SRp55 upon endogenous G6PD expression most likely indicates its importance in other regions of the pre-mRNA because it did not bind to the splicing element in the RNA affinity assay (Fig. 1A). Depletion of SRp20 or the other SR proteins did not affect the expression of cyclophilin B, a negative control, indicating a specific effect upon splicing of G6PD mRNA.
**SRp20 enhances splicing of exon 12-containing mRNA.**

To verify that SR proteins and, specifically, SRp20 are direct enhancers of G6PD splicing, we tested SR protein function in the *in vitro* splicing assay. The G6PD pre-mRNA substrate containing exon 11, intron 11, and exon 12 (IVS 11-12) splices inefficiently (9%) as compared to 22% splicing with another G6PD splicing substrate containing exon 8, intron 8, exon 9 (IVS8-9; Fig. 4A). This is due to the strong ESS within the regulatory element of exon 12 (15). To restrict the overriding effect of the ESS on splicing in this assay, we used a limiting amount of HeLa nuclear extract, which reduced the amount of silencing proteins (hnRNPs) and SR proteins contributed by the nuclear extract. This limiting amount of nuclear extract didn’t support splicing compared to the full complement of nuclear extract (Fig. 4B, compare lanes 1 and 2 of both panels) and, thus, provided a baseline to which SR proteins were added to test for their ability to enhance splicing. Addition of a mixture of SR proteins to the assay enhanced the splicing of both IVS 8-9 and IVS 11-12 (Fig. 4B, compare lanes 2 and 3 of both panels). SR proteins enhanced splicing of the exon 12-containing substrate by 3-fold. An apparent decrease in the substrate RNA was consistently observed when the extent of splicing in the assay was low. As this pattern of bands was observed numerous times, the differences in substrate mRNA between lanes cannot be attributed to loading errors; therefore quantitation of the spliced product without correction by the substrate RNA band was appropriate. We attribute this decrease to degradation of the substrate in the absence of splicing. This may have involved non-specific actions of RNase or may reflect targeted degradation of the intron-containing transcript by the nuclear exosome (30). Alternatively, the differences in intensity of the pre-mRNA observed between lanes may be attributed to the occurrence of two bands of similar size when splicing was observed, one corresponding to unspliced substrate and the other corresponding to the intron lariat attached to the 3’ exon, an intermediate in the splicing reaction.

Because SRp20 binds to the regulatory element and knock-down of SRp20 inhibits G6PD expression, we asked if SRp20 *per se* could enhance splicing of IVS 11-12. SRp20 was purified from Sf9 cells following baculovirus infection. The presence of SRp20 was confirmed with western analysis (Fig. 4D), and it comprised 36% of the total
protein as detected with Coomassie staining (data not shown). Addition of SRp20 increased splicing of IVS 11-12 by 2-fold or more (Fig. 4C). To confirm that SRp20 caused the increase and not co-purifying Sf9 proteins, a control protein mixture was added to the splicing assay. This mixture is a protein preparation from Sf9 cells that were infected with a baculovirus expressing SC35, but purification of SC35 was unsuccessful. As such, SC35 constituted less than 5% of the total protein of this mixture (data not shown). The control protein mixture did not enhance splicing of either substrate (Fig. 4C, lane 4 of both panels). In contrast to the specific effect of SRp20 on IVS11-2 splicing, it did not enhance splicing of the IVS 8-9 RNA substrate (Fig. 4C, lanes 2 and 3 of left panel). Thus, splicing activation by SRp20 is sequence specific. In sum, SRp20 appears to be a necessary splicing activator, enhancing expression of G6PD and doing so through the exon 12 regulatory element.

**Phosphorylation of SR proteins changes with insulin and arachidonic acid treatment.**

We have determined that refeeding enhances the binding of SRp20 to the regulatory element. We sought to further understand how nutrients and hormones might affect SR protein activity and contribute to the regulated binding of SRp20 in the liver. Phosphorylation of the RS domain of SR proteins is an essential step regulating SR protein activity (31). The goal of the current experiment was to determine if there was a change in the phosphorylation status of nuclear SR proteins due to hormones and nutrients. Treatment of primary rat hepatocytes with insulin and a high glucose medium reflects the humoral *milieu* of the refed state (32). While glucagon is a hormone associated with metabolic regulation during starvation, it does not decrease expression of G6PD (33). Starvation is also accompanied by lipolysis and an increase in circulating free fatty acids (34). Treatment of hepatocytes with the non-esterified fatty acid, arachidonic acid, is our model for starvation-induced lipolysis. Fatty acids cause a decrease in G6PD expression in the hepatocytes, and the time course of this decrease reflects the decrease in G6PD expression during starvation (14, 17). Changes in G6PD mRNA abundance by these treatments are caused by changes in the splicing of the primary transcript (13).
Incubation of rat hepatocytes with insulin and high glucose medium significantly enhanced the amount of phosphorylated SRp20, SRp30a/b, and SRp40 in nuclear extracts as compared to media alone (Figs. 5 B and C). The increase in phosphorylation of these proteins coincided with a 3-fold increase in G6PD mRNA abundance (Fig. 5A). There was a trend toward an increase in phosphorylation of SRp55 and SRp75 in response to insulin, but it did not reach the level of significance. In contrast to the effect of insulin alone, addition of arachidonic acid to the medium significantly attenuated the amount of phosphorylated SRp40 and SRp20 by 50-80%. This decrease in SR protein phosphorylation coincided with a 50% decrease in G6PD mRNA abundance. Equal amounts of protein were used for the analysis as indicated by the control protein, lamin A/C (Fig. 5B). Notably, insulin increased and arachidonic acid decreased the amount of phosphorylated SRp20 to a greater extent than any other SR protein. The large changes in phosphorylation are consistent with a role for this protein in nutrient-induced splicing regulation. This change in the amount of phosphorylated SR proteins in response to insulin and arachidonic acid may reflect a phosphorylation/dephosphorylation switch, a change in expression of these SR proteins, or movement of SR proteins to and from the nucleus. Regardless, this experiment indicates that insulin and arachidonic acid modulate the nuclear content of phosphorylated SR proteins, which enhances their regulatory capacity in the splicing process. Furthermore, the changes in SRp20 content coincide with regulation of splicing of G6PD mRNA.

Discussion

Understanding how external signals such as nutrients and hormones affect the splicing process introduces a new paradigm for regulating cellular function. Because the majority of genes undergo alternative splicing, this process is central in determining the protein composition of cells. External stimuli and hormones can exert an effect upon splicing by increasing or decreasing the expression of splicing factors and/or by changing their activity (8, 10, 35, 36). G6PD was one of the first pre-mRNAs identified to be regulated at the step of splicing by nutritionally-responsive hormones and nutrients (37). In this report, we’ve identified SR proteins as targets in the nutrient regulation of
splicing. SR proteins bind to the exon 12 regulatory element of G6PD pre-mRNA in intact liver, and refeeding enhances their binding. Of the SR proteins, SRp20 enhances the splicing of G6PD mRNA, and loss of SRp20 decreased G6PD expression. Furthermore, the phosphorylation state of SRp20 is regulated by nutrients and the nutritionally regulated hormone, insulin. Therefore, SRp20 is a candidate splicing activator for the nutritional regulation of G6PD expression.

SRp20 has defined roles in directing alternative splicing as well as mRNA transport to the cytosol (38-40). Our data suggest that SRp20 is having a direct effect upon splicing of G6PD mRNA rather than upon transport alone because it enhanced removal of intron 11 within the in vitro splicing substrate IVS 11-12 (Fig. 4C). The inefficient splicing of exon 12-containing substrates is caused by an inhibition of the formation of the A complex (15). This intermediate in spliceosomal catalysis involves the recruitment of U2 snRNP to the branch point and formation of a stable interaction between U2 snRNP and the mRNA (41). SR proteins can enhance spliceosomal formation by recruitment of U2 snRNP (42). This suggests that SR proteins would be required for G6PD splicing. Thus, SRp20 binding to exon 12 and activation of splicing in vitro is consistent with this role of SR proteins in spliceosome formation.

The binding of SRp20 to the regulatory element would not be predicted based on the presence of a canonical binding site. SRp20 binding sites that have been identified are CU and CA rich (43-45). Current computer ESE prediction software do not identify SRp20 binding sites within the regulatory element encompassing nucleotides 43-72 of the 93 base exon 12, but putative SRp20 binding sites conforming to known consensus sequences occur just 5’of the regulatory element and overlapping the 3’ end of the element (Splicing Rainbow, Morais and Valcarcel; ESE finder (46, 47)). In contrast, this same software predicts binding of proteins such as SRp40 and SRp55; binding of these proteins was not detected in our assays despite their abundance in the nuclear extract (Fig. 1C). ESE prediction software uses sequences determined by SELEX or bioinformatics to establish the comparison sequence and are, thus, isolated from the complexity of the cellular environment. Thus, these predictions must be followed up with functional analyses. The global diversity of SRp20 binding sites has not been established
experimentally. Within the G6PD regulatory element, CA rich regions appear to be sufficient to support binding.

Most remarkable is the large change in SR protein binding with refeeding. The increased binding was observed in intact liver using ChIP. This is the first demonstration of regulated binding of SR proteins to mRNA in response to nutritional status. The specific interactions of SRp20 and SRp75 with the G6PD regulatory element were determined in vitro using an RNA affinity assay. While nutritional status altered the binding of both SRp20 and SRp75 to the splicing regulatory element, only the binding of SRp20 was relevant for increased splicing of G6PD mRNA. In this regard, knock-down of SRp75 failed to decrease expression of G6PD mRNA (Fig. 4C). The involvement of SRp20 in the nutritional regulation of splicing is further supported by the regulation of SRp20 phosphorylation in response to insulin and arachidonic acid. Upon phosphorylation, SR proteins move from the cytoplasm to the nucleus and from sites of concentration within the nucleus, called speckles, to nascent RNA at transcription sites (7). In addition, phosphorylation enhances binding to RNA regulatory elements and protein-protein interactions (31, 48). SR proteins bound to the G6PD regulatory element were phosphorylated based on their detection with a phosphorylation-specific antibody (Fig. 2). This binding of SR proteins in the fed state is consistent with our earlier data demonstrating the opposite pattern of binding by splicing silencing proteins during starvation. Members of the hnRNP family of splicing silencing proteins, K, L and A2/B1, bind to the G6PD regulatory element in the starved state, and this binding is decreased by refeeding (16). Competition between SRp20 and splicing silencers has been demonstrated in regulation of the alternative splicing of the insulin receptor (40). A similar competition for binding may be part of the regulatory mechanism controlling G6PD splicing in response to nutrients and nutritional status.

A considerable body of literature supports a broad role of insulin in alternative splicing. Splicing-sensitive microarrays performed with drosophila cells demonstrate that greater than 150 genes undergo alternative splicing in response to insulin treatment (49). In mammalian cells, insulin regulates alternative splicing of PKCβII (50). The effect of insulin upon alternative splicing of PKCβII is coincident with an increase in the phosphorylation of SRp40 (9). Insulin-mediated increases in SRp40 activity involve both
direct phosphorylation by Akt2 and indirect mechanisms via phosphorylation of Clk/Sty, an SR protein kinase, by Akt2 (10). The coincident increase in G6PD splicing and SR protein binding with refeeding likely involves a regulatory role for insulin because insulin is required for the diet-mediated increases in G6PD expression (37). In hepatocytes in culture, insulin increases G6PD mRNA and increases SR protein phosphorylation, including phosphorylation of SRp20.

Previous evidence from our lab demonstrates that the increase in splicing of G6PD that we observe with insulin treatment is due to an increase in its signaling through PI3K and Akt; whereas arachidonic acid treatment attenuates insulin’s signaling by inhibition of PI3K activity (51). Arachidonic acid decreases the amount of phosphorylated SR proteins and causes a coincident decrease in the accumulation of G6PD mRNA (Fig. 5). In particular, these changes were observed with SRp20, the SR protein most relevant for regulating splicing of G6PD. The presence of a consensus sequence for Akt phosphorylation in the RS domain of SRp20 suggests that this protein can be a target of insulin action directly. Insulin action could also be indirect via stimulation of Clk/Sty. Consistent with this concept, the phosphorylation-specific antibody used in these studies detects phosphorylations by SR protein kinases including Clk/Sty, suggesting an increase in their activity. Identifying the relevant kinases involved in SR protein phosphorylation in intact liver and primary rat hepatocytes cultures is the subject of on-going investigations in the laboratory. The decrease in phosphorylation of SR proteins during arachidonic acid treatment may not be the result of reduced kinase activity alone, but alternatively, may be due to an increase in phosphatase activity during this treatment. The bioactive lipid, ceramide, reduces phosphorylation of SR proteins via activation of PP1 (52), and arachidonic acid has been shown to induce ceramide production from sphingomyelin in HL-60 cells (53). The findings that insulin enhances the amount of phosphorylated SR proteins in the nucleus of hepatocytes and that refeeding enhances the binding of SR proteins to this nutritionally regulated target are significant intrinsically. SR proteins enhance the splicing of multiple genes, and, thus, nutritional status could impact the splicing of many transcripts in addition to G6PD.

In sum, these data indicate that nutrients and nutritional status induce changes in the activity of SR proteins, which impacts the splicing of a model metabolic gene. These
data provide further support for the role of insulin in pre-mRNA processing, providing another layer of control over gene expression beyond its well-described effects upon transcription. We hypothesize that other pre-mRNA transcripts are regulated by nutrients at the step of splicing, as nutrient signaling affects the abundance of nuclear phosphorylated SR proteins and the binding capacity of SR proteins. These results have important implications to fully understand the process by which our metabolic organs respond appropriately to nutrients and hormones and provide a new paradigm for nutrient regulation of cellular function.

Materials and Methods

All animal experiments were conducted in conformity with the Public Health Service policy on Humane Care and Use of Laboratory Animals, additionally; the Institutional Animal Care and Use Committee of the Division of Laboratory Animal Resources at West Virginia University approved all experimental procedures.

Animal care and liver nuclear extract preparation - Male Sprague-Dawley rats (200-300 g) were either fasted for 24 h or fasted for 24 h and then refed a fat free/high glucose diet containing 1% safflower oil as a source of essential fatty acid (fat free/high glucose USB diet, #1810092, Purina Mills) for 16 h prior to sacrifice. Liver nuclear extracts were prepared by the method of Schibler et al. (54) with modifications. Briefly, liver nuclei were isolated by immediately homogenizing the livers with a Thomas homogenizer in buffer I (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 50 mM NaF, 1 mM benzamidine, 0.5 µg/ml leupeptin, 2 µg/ml aprotinin, 10 mM β-glycerophosphate, and 0.1 mM Na$_2$MoO$_4$). The homogenate was layered over a cushion of buffer II (buffer I containing 0.75 M sucrose), and centrifuged for 10 min at 100 x g to produce a crude nuclear pellet. The pellet was further homogenized in buffer III (buffer I except 2 M sucrose, 0.1 mM EDTA, and 0.1 mM EGTA). This homogenate was layered over buffer III, topped with mineral oil, and centrifuged 1h at 155,000 x g. Nuclear proteins were extracted from
nuclei as per the Dignam protocol (55) in extraction buffer containing 20 mM Hepes, pH 7.9, 25% glycerol, 350 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and the same concentrations of protease and phosphatase inhibitors used in buffer I and stored at -80°C until use.

**RNA affinity assay** - RNA oligonucleotides corresponding to nucleotides 43-72 and 79-93 of exon 12 of G6PD were purchased from IDT. One thousand pmol of each RNA oligonucleotide was attached to adipic acid dihydrazide-agarose beads (Sigma) using our modification (16) of the methods of Langland et al. (56) and Caputi et al. (57). Each RNA-bead complex (250 µl) was mixed with starved or refed rat liver nuclear extract in a binding reaction containing 250 µg of protein in 500 µl of buffer (20 mM Hepes, pH 7.4, 9% glycerol, 70 mM KCl, 0.2 mM EDTA pH 8.0, 1000 ng/ml tRNA, 2.5 mM ATP, 2 mM MgCl₂, and 0.2 mM DTT). The binding reactions were incubated at 30°C for 30 min, after which, the bead mixtures were placed into columns (5-mL plastic columns with fritted ends), flow-through was eluted with gravity drip, the RNA-bead complexes were washed with 500 µl of wash buffer containing no salt (20 mM Hepes, pH 7.4, 5% glycerol, 0.2 mM EDTA pH 8.0, and 0.1 mM DTT), and proteins were eluted with gravity drip in a step-wise manner with 1 ml volumes of wash buffer, as listed above, containing an increasing concentration of KCl (100 mM, 150 mM, 250 mM, 500 mM, 750 mM, 1000 mM). Each elution was collected into a separate tube and concentrated and de-salted with Amicon® Ultra centrifugal filter devices (3,000 MWCO, Millipore). Equal volumes (50% of total volume) of concentrated protein from each sample were separated on 10% SDS-PAGE gels, transferred to PVDF membranes (Bio-Rad), and probed with SRp20 antibody (Zymed) or 16H3 antibody (Zymed), which detects SRp75, SRp55, SRp40 and SRp20. After incubation with secondary antibodies conjugated to horseradish peroxidase (Bio-Rad), signals were detected with ECL Plus (GE Healthcare), and visualized on a Typhoon 9410 Imager (GE Healthcare) and with X-ray film.

**Animal care and chromatin immunoprecipitation (ChIP) assay** - Six-week old C57BL/6 mice were fed a high carbohydrate diet (described above, Purina Mills) for one week. Mice in the starved group were fasted for 18 h prior to sacrifice. Mice in the refed group
were fasted for 18 h, refed the high carbohydrate diet for 12 h, and then sacrificed. The crosslinking reaction and ChIP assay are modifications of existing protocols (58, 59). Immediately after sacrifice, the livers were removed and immersed in four volumes of phosphate buffered saline containing 1.25% formaldehyde, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 50 mM NaF, 1 mM benzanidine, 0.5 µg/ml leupeptin, 2 µg/ml aprotinin, 10 mM β-glycerophosphate, 30 mM p-nitrophenyl phosphate, and 0.1 mM Na$_2$MoO$_4$, minced, and mixed by rotation for 12 min at room temperature. The crosslinking reaction was stopped with the addition of glycine to a final concentration of 125 mM. Samples were homogenized in a dounce homogenizer (8 strokes, loose pestle), incubated 15 min on ice, and then centrifuged 5 min at 1500 x g at 4°C. The pellets were resuspended in 3 volumes of cell lysis buffer (5 mM Hepes, pH 8.0, 85 mM KCl, 0.5% NP40, and protease and phosphatase inhibitors as listed above), homogenized in a dounce homogenizer (15 strokes, loose pestle), incubated on ice for 15 min, and centrifuged at 3500 x g for 5 min at 4°C. The pellets were resuspended in 1 volume of nuclear lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.1, 10 mM EDTA, and protease and phosphatase inhibitors as listed above), homogenized with a dounce homogenizer (5 strokes, loose pestle), aliquoted into 1-ml volumes, and incubated on ice for 10 min. Samples were sonicated with continuous pulse (output 2) in 4 x 15 sec bursts. The probe was chilled between each pulse. Samples were then cleared by centrifugation at 19,000 x g for 10 min, supernatants were reserved and diluted 5-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 16.7 mM MOPS, pH 7.3, 1.2 mM EDTA, 167 mM NaCl, and the same concentrations of protease and phosphatase inhibitors as listed above), and stored at -80°C until immunoprecipitation steps.

For the immunoprecipitation, protein A/G agarose beads (Santa Cruz) were washed 5 times in dilution buffer and blocked in dilution buffer containing 5 mg/ml BSA and 250 µg/ml sonicated salmon sperm DNA. An aliquot from each ChIP sample was removed prior to immunoprecipitation to serve as the “input”. ChIP samples were then pre-cleared with blocked agarose beads for 2 h with rotation, centrifuged to pellet the beads, and supernatants were placed into fresh tubes to which the following immunoprecipitation antibodies were added (7.5 to 15 ug): phosphorylated SR (1H4, Zymed), hnRNP M (Zymed), and RNA polymerase II (Covance). The “no antibody”
control was treated identically to immunoprecipitations except it received no primary antibody. Samples were immunoprecipitated overnight with rotation at 4°C, after which samples were rotated with blocked beads for 2 h, and washed once with each of the following buffers (1 ml of each): low salt buffer (20 mM MOPS, pH 7.3, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), high salt wash buffer (20 mM MOPS, pH 7.3, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton-X 100), LiCl wash buffer (10 mM MOPS, pH 7.3, 1 mM EDTA, 0.25M LiCl, 1% NP40, 1% deoxycholate), and finally 2 times in a buffer containing 10 mM MOPS, pH 7.3 and 1 mM EDTA. All wash buffers contained protease and phosphatase inhibitors as listed above. The immunoprecipitated proteins were eluted from the agarose beads with 0.1M NaHCO$_3$, 1% SDS.

Immunoprecipitated and input samples were reverse crosslinked with 200 mM NaCl at 65°C for 6 h, digested with proteinase K (180 µg/ml) for 1 h at 45°C, phenol/chloroform extracted, ethanol precipitated, and resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA.

Quantitative real-time PCR was performed with QuantiTect probe PCR reagents (Qiagen) with the following primers and dual-labeled probes (IDT): G6PD exon 12 regulatory element (F: 5’-ACCTCCATCCTACACTGTATCC-3’, R: 5’-CTGTTAGCTTCCATCCTCCCT-3’, probe: 5’/-56-FAM/TGGCGTATCTTCACACCCTGCTGCACAA/3BHQ1/-3’)) and PEPCK (F: 5’-TTCGTGGAAGGCAATGCTCA-3’, R: 5’-TCTTCAGCTTGGGATGACA-3’, probe: 5’/-5IAbFQ/TATATCCACATCTGCGATGGCTCCGAGGA/36-FAM/-3’). The amount of DNA that was immunoprecipitated relative to the amount present in total input chromatin was determined with the following formulas: $\Delta Ct=Ct(input)-Ct(IP)$, % total = $2^{\Delta Ct} \times 1.7$, where 1.7% is the percent that the input chromatin represents of the total chromatin (60). This value was then normalized to the signal found in the “no antibody” control.

**siRNA transfection** - One day after passage, HeLa cells (2 X 10$^5$) were transfected with 75 nM siRNA pools using 7.5 µl of TransIT-siQUEST® Transfection Reagent (Mirus) in MEM containing 10% FBS. All siRNAs targeting a particular mRNA were siGENOME SMARTpools (Dharmacon) consisting of 4 siRNA duplexes. The siRNA pool for SRp20 contained the following duplexes: GAGUGGAACUCUGUCGAUUG,
GGACUGUAGGUAAGUUGAUGUA, CGAAGUGUGUGGGUUGCUA, CGAGAUGAUUAUCGUAGGA. The siRNA pool for SRp55 consisted of the following duplexes: GCAGAAUAUUAGGCUCUA, GCGACAAGCAGUGAAGUA, CGUACAGAAUACAGGCUCUA, GGAUACAGCAGUCGAGAA. The siRNA pool for SRp75 contained the following duplexes: GGCAAGACCUAAAGGAUUA, GAAGUGGCCGAGAUAAAUA, GAAUCACGCUCCAGAUCAA, GCAAAGACCAAGCUGAAGA. SiGENOME non-targeting siRNA pool #1 was used as the negative control. Media was changed 24 h after siRNA transfection. The cells were incubated an additional 24 h and then were transfected with 3 µg of the splicing reporter, pβ-gal ex12-ex13 using 6 µl of TransIT®-LT1 Transfection Reagent (Mirus) in complete MEM. Twenty-four h later, total RNA and whole-cell protein extracts were collected. RNA was extracted with TRI Reagent® (Applied Biosystems), DNase digested, and expression of the splicing reporter or endogenous mRNAs was quantified by real-time RT-PCR (ICYCLER, Bio-Rad) using the QuantiTect SYBR Green kit (Qiagen). Real-time RT PCR primers were as follows: pβ-gal ex12-ex13 (F: 5’-ACGACTCCTGGAGCCCGTCA-3’, R: 5’-CTGTGGGCGCCGCTGC-3’), human cyclophillin B (F: 5’-GCACAGGAGAAAGAGCATC-3’, R: 5’-CTTCTCCACCTCGATTTGC-3’), human G6PD (F: 5’-GCTGAGATTTTGCCAACAGG-3’, R: 5’-GCATCAGTCCCCGGATGA-3’). The amount of each mRNA was calculated using a relative standard curve. Whole-cell protein extracts were collected in lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Triton-X 100, 1 mM Na3VO4, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSF, and a mixture of protease inhibitors (Complete Mini, Roche). Equal amounts of protein (2.5 µg) were separated on 10% SDS-PAGE gels, transferred to PVDF membranes (Bio-Rad), and probed with SRp20 antibody (Zymed), culture supernatant from hybridoma cells expressing mAb104 (ATCC), or Lamin A/C antibody (Cell Signaling). After incubation with secondary antibodies with horseradish peroxidase conjugation, signals were enhanced with ECL Plus (GE Healthcare), visualized on a Typhoon 9410 Imager (GE Healthcare), and quantified with ImageQuant TL (Molecular Dynamics).
In vitro splicing assays - In vitro splicing assays were performed as described previously (15) with slight modifications. Briefly, the positive control splicing assays contained 60% (v/v) HeLa nuclear extract (Promega), and splicing complementation experiments contained a limiting amount of nuclear extract (10%) to which the remaining volume was replaced with buffer D (20 mM Hepes-KOH, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 5% glycerol, 0.5 mM PMSF, and 0.5 mM DTT) or SR proteins, which were suspended in buffer D. The appropriate volume for the limiting amount of nuclear extract was empirically determined based on the volume that demonstrated negligible splicing in the absence of supplemented SR proteins (data not shown). All components of the buffer were maintained constant across all splicing reactions. Quantitation of the % splicing in the in vitro splicing assays in Fig. 3A, was performed as described previously (15) using ImageQuant where the splice product was divided by the sum of the splice product and the pre-mRNA signal. Quantitation of the relative level of splicing in Fig. 3B and 3C consisted of measuring the intensity of the spliced product with ImageQuant, which was then normalized to the splice product observed with the positive control (full complement of nuclear extract).

The mixture of SR proteins was purified from HeLa cells via the method of Zahler (61). Purified SRp20 containing a His tag was obtained from Sf9 cells infected with an SRp20 baculovirus (a gift from J.G. Patton (62) and prepared by Virusys Corporation), and the preparation consisted of 36% SRp20. The control protein mixture was an impure protein preparation from Sf9 cells infected with SC35 baculovirus; this preparation contained a very low amount of SC35 (<5% of total protein) as a result of problems in the purification procedures (data not shown). The preparation controls for the addition of Sf9 proteins per se to the splicing reaction. To determine the purity of each protein preparation, an aliquot was separated on a 12% SDS-PAGE gel, stained with colloidal Coomassie (Invitrogen), and quantified with ImageJ (NIH). Western analysis was also performed upon the purified SRp20 sample with the SRp20 antibody (Zymed).

Hepatocyte culture and nuclear extract preparation - Male Sprague-Dawley rats (150-250 g) were maintained on a chow diet until they were fasted 16 h prior to isolation of
hepatocytes via the method of Seglen as previously described (14). Hepatocytes (3 x 10^6) were plated in collagen-coated 60 mm dishes containing Hi/Wo/BA medium (Waymouth MB752/l plus 20 mM Hepes, pH 7.4, 0.5 mM serine, 0.5 mM alanine, 0.2% bovine serum albumin) plus 5% newborn calf serum (37 °C, 5% CO_2). After 4 hr, the cells were washed and plated in the same media lacking serum, and a matrigel overlay was added (0.3 mg/ml; BD Pharmingen). Sixteen h later, the media was replaced with fresh media alone or media containing 80 nM insulin or 80 nM insulin plus 175 uM arachidonic acid conjugated to bovine serum albumin (4:1 ratio; 63). Twelve h later, the media was replaced with media of the same composition. Twenty-four h after the initial treatment, nuclear extracts (from 6-12 plates per treatment) were prepared via the method of Dignam et al. (55), and western analysis was performed as described above and quantified with ImageQuant TL (Molecular Dynamics) and ImageJ (NIH). At the same time point, total RNA was collected as described above under “siRNA transfection”. Real-time RT PCR was performed with QuantiTect probe PCR reagents (Qiagen) with the following primers and dual-labeled probes (IDT): G6PD (F: 5’-TATGTCTATGGCAGCGAGGT-3’, R: 5’-GCAGAGTGCGAGATGGTGAAG-3’; G6PD probe, 5’-FAM/CCACAGAGGCAGATGCTGATGAAGAA/3’BHQ-3’) and β-actin (F: 5’-GATGACCCAGATCATGTGTGAGACC-3’ R: 5’-GGAGTCCATCACAATGCGACC-3’, probe: 5’-HEX/CCCAGCCATGTAGCTGACCAGTGAGAAG/3BHQ-3’). Cyclophilin B expression was detected with the Qiagen Quantitect SYBR Green reverse transcription-PCR system and the following primers: (F: 5’-CGTGGGCTCCGTTCTT-3’, R: 5’-TGACTTTAGGTCCCTTCTTCTTATC-3’). Cyclophilin B and β-actin were used as endogenous reference genes, and the amount of G6PD was calculated using relative standard curves.

Statistical Analyses – For Figs. 2 and 3C, statistical comparisons were made using Student’s t-test, and significance was defined as P < 0.05. For 5C, one-way ANOVA was performed, and, if the overall P-value was significant (P < 0.05), Dunnett’s Multiple Comparison Test was performed to make pairwise comparisons to the insulin treatment
(media alone vs. insulin and insulin vs. insulin + arachidonic acid; GraphPad Prism, version 4.0).

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Figure legends:

Figure 1. Refeeding enhances the binding of SRp20 and SRp75 to the splicing regulatory element. An RNA affinity assay was performed with the following RNA oligonucleotide-bead complexes: regulatory element (nucleotides 43-72 of exon 12 of G6PD), control region (nucleotides 79-93 of exon 12 of G6PD), and beads alone which were used to pull-down proteins from nuclear extracts prepared from livers of starved and refed rats. Proteins were eluted in buffers containing an increasing concentration of KCl as indicated with the triangle, and proteins were detected with immunoblots. (A) Immunoblot with 16H3 antibody; the protein marker (kDa) is indicated to the right of each gel. I, input representing 2.5 µg of nuclear protein. (B) Immunoblot with SRp20 antibody. (C) The protein inputs (10 µg) used in the affinity assay detected with 16H3 antibody. Due to the low abundance of SRp20 when detected with 16H3, another blot with 20 µg of nuclear extract is shown beneath. SR proteins are identified by size and indicated on the right. Each RNA affinity assay is a representative of n = 3 assays performed using two independent nuclear extract preparations.

Figure 2. Refeeding increases the binding of phosphorylated SR proteins to the regulatory element of G6PD in vivo. Chromatin immunoprecipitation assay (ChIP) was performed in livers of starved and refed mice with the following antibodies: phosphorylated-SR protein (phospho-SR), hnRNP M, and RNA polymerase II (Pol II). The occupancy of each of these proteins upon exon 12 of the G6PD mRNA and exon 2 of the PEPCK mRNA was measured using real-time PCR. The values for each are the amount on the test sequence relative to the “beads alone” control within each dietary group. The “*” symbol indicates P < 0.05. Repetitions were as follows for G6PD: IP of phospho-SR n=3; IP of hnRNP M n=3; IP of Pol II n=2; and for PEPCK: IP of phospho-SR n=5; IP of hnRNP M n=3; IP of Pol II n=2.

Figure 3. SiRNA-mediated depletion of SRp20 reduces the splicing of a G6PD reporter and the endogenous G6PD mRNA. (A) Whole cell lysates were prepared from HeLa cells 24 h after transfection with siRNA pools targeting SRp20, SRp55, SRp75, as well as the
non-targeting (NT) control. Western analysis was performed with antibodies against multiple SR proteins (mAB104), SRp20, and lamin A/C. (B) Schematic representation of the pβ-gal ex12-ex13 splicing reporter. The CMV promoter drives expression of the reporter RNA. Mouse DNA containing exon 12, intron 112 and exon 13 through the end of the G6PD gene was ligated to β-galactosidase. (C) The expression of the transiently transfected G6PD reporter (pβ-gal ex12-ex13), endogenous G6PD gene, and cyclophillin B gene was measured by real-time RT-PCR following knock-down with SRp20, SRp55, SRp75, and non-targeting (NT) siRNA. The “∗” indicates a significant difference (P < 0.05). Repetitions were as follows: pβ-gal ex12-ex13 expression following treatment with SRp20 siRNA n=6, SRp55 siRNA n=9, SRp75 siRNA n=9; endogenous G6PD expression following treatment with SRp20 siRNA n=12, SRp55 siRNA n=9, SRp75 siRNA n=9; endogenous cyclophillin B expression following treatment with SRp20 siRNA n=6, SRp55 siRNA n=9, SRp75 siRNA n=9.

**Figure 4.** SRp20 enhances splicing of RNA containing the exon 12 regulatory element. (A) *In vitro* splicing assays were performed using the RNA substrates: IVS 8-9 (exon 8, intron 8, and exon 9) and IVS 11-12 (exon 11, intron 11, exon 12) and the maximum amount of HeLa nuclear extract. The “∗” indicates the spliced product. A representative assay is shown in which all samples were run on the same gel, but intervening lanes were removed from the image; this experiment has been repeated multiple times. I, input; NE, nuclear extract (B) *In vitro* splicing assays were performed utilizing IVS 8-9 and IVS 11-12 and a mixture of purified SR proteins. The following treatments were used: maximum amount of nuclear extract (lane 1), limiting nuclear extract as described in the text (lane 2), limiting nuclear extract plus the mixture of purified SR proteins (lane 3). A representative gel is shown. To the right of the gel are cartoons indicating the location of the unspliced substrate and the spliced product. Below each gel is the relative level of splicing, which is the amount of spliced product normalized to the positive control (lane 1) and is an average of n = 3 independent splicing assays. (C) *In vitro* splicing assay of IVS 8-9 and IVS 11-12 were performed utilizing purified SRp20. The following treatments were used: maximum amount of nuclear extract (lane 1), limiting nuclear extract (lane 2), limiting nuclear extract plus SRp20 (lane 3) and limiting nuclear extract
plus control Sf9 protein mixture (lane 4). Below each gel is the relative level of splicing, which is the amount of spliced product normalized to the positive control (lane 1) and is an average of n = 3 independent splicing assays. (D) Immunoblot of the purified His-tagged SRp20 produced in Sf9 cells detected with the SRp20 antibody.

**Figure 5.** Insulin and arachidonic acid regulate the amount of phosphorylated SR proteins in the nuclei of primary rat hepatocytes. (A) Primary rat hepatocytes were incubated in high glucose (27.5 mM) medium alone (NA), insulin (I; 80mM), or insulin plus arachidonic acid (I+AA; 175 µM). After 24 h, total RNA was isolated and the amount of G6PD mRNA was measured by real-time RT-PCR. The value for the amount of G6PD mRNA with insulin treatment (1.5 ± 0.3) was set at 1 and the values for the NA and I+AA treatments are expressed relative to the insulin treatment. The data represent n= 3 independent hepatocyte isolations. (B) Nuclear extract was prepared from the hepatocytes treated as described in part A. The extracts were subjected to western analysis using an antibody against phosphorylated SR proteins (mAB104) and lamin A/C. A representative immunoblot is shown. The identities of the SR proteins are listed on the left side of the gel. The “<” symbol indicates the dye front of the gel. (C) Quantitation of the immunoblot data from n = 4 independent experiments. The amounts of the phosphorylated SR proteins in each treatment were measured by densitometry and are indicated on each graph by arbitrary densitometric units (Arb. Dens. U.). The “*” symbol indicates a significant difference (P < 0.05) between NA vs. I treatments or I vs. I + AA treatments.
Figure 1

A. IB: 16H3

RNA oligos
Regulatory element
Control region
Beads
Starved Refed

B. IB: SRp20

RNA oligos
Regulatory element
Control region
Beads
Starved Refed

C. IB: 16H3

kDa S R
150
100
75
50
37
25
20
SRp75
SRp55
SRp40
SRp20
SRp20
Figure 2
Figure 3

A. | siRNA |
---|-------|
NT | SRp20 | NT | SRp55 | NT | SRp75 |
SRp75 |   |   |   |   | IB: mAb104 |
SRp55 |   |   |   |   | IB: SRp20 |
SRP40 |   |   |   |   | IB: Lamin A/C |
SRP30a/b |   |   |   |   | |
SRP20 |   |   |   |   | |
Lamin A/C |   |   |   |   | |

B. CMV → β-gal 12 → 13 → 3′ UTR

C.

1. β-gal in 12 or 13
2. Endogenous GAP43 Relative Level
3. Cyclophilin B Relative Level

Legend:
- NT siRNA
- SRp20 siRNA
- SRp55 siRNA
- SRp75 siRNA

* Indicates statistically significant difference compared to NT siRNA
Figure 4

A.

B.
Figure 4 (continued)
Figure 5

A. G6PD mRNA Relative Level

B. Insulin + +
Arach. acid - - +
SRp75
SRp55
SRp40
SRp30a/b
SRp20
Lamin A/C

C. SRp75
SRp55
SRp40
SRp30a/b
SRp20

Arb. Dens. U.

NA I I + AA

NA I I + AA

NA I I + AA

NA I I + AA

NA I I + AA

Na H. Hagen