Published in final edited form as:

Biochim Biophys Acta. 2006 ; 1759(11-12): 552–561.

Identification of hnRNPs K, L and A2/B1 as candidate proteins involved in the nutritional regulation of mRNA splicing

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Summary

Nutrient regulation of glucose-6-phosphate dehydrogenase (G6PD) expression occurs through changes in the rate of splicing of G6PD pre-mRNA. This posttranscriptional mechanism accounts for the 12- to 15-fold increase in G6PD expression in livers of mice that were starved and then refed a high-carbohydrate diet. Regulation of G6PD pre-mRNA splicing requires a cis-acting element in exon 12 of the pre-mRNA. Using RNA probes to exon 12 and nuclear extracts from livers of mice that were starved or refed, proteins of 60 kDa and 37 kDa were detected bound to nucleotides 65–79 of exon 12 and this binding was decreased by 50% with nuclear extracts from refed mice. The proteins were identified as hnRNP K, and L, and hnRNP A2/B1 by LC-MS/MS. The decrease in binding of these proteins to exon 12 during refeeding was not accompanied by a decrease in the total amount of these proteins in total nuclear extract. hnRNPs K, L and A2/B1 have known roles in the regulation of mRNA splicing. The decrease in binding of these proteins during treatments that increase G6PD expression is consistent with a role for these proteins in the inhibition of G6PD mRNA splicing.

Keywords

RNA splicing; hnRNP; nutritional regulation; posttranscriptional gene regulation; lipogenesis; liver

1. Introduction

The conversion of excess dietary energy to stored fuel via de novo fatty acid synthesis is essential to energy homeostasis. Excess carbohydrate and protein in the diet are the primary substrates for this pathway, which is most active in liver and adipose tissue. Fatty acid synthesis involves a family of enzymes commonly referred to as lipogenic enzymes [1]. Lipogenic enzymes include glucose-6-phosphate dehydrogenase (G6PD), ATP-citrate lyase, malic enzyme, acetyl-CoA carboxylase, and fatty acid synthase. Consistent with their role in energy metabolism, the activities of these enzymes are induced when animals are fed a high-carbohydrate diet and decreased during starvation or by the addition of polyunsaturated fat to the diet. The unique aspect of this dietary regulation is that the nutrients per se play a significant role in the molecular mechanisms regulating the synthesis of these enzymes and in the signal

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transduction pathways that mediate the change in nutritional status. While ATP-citrate lyase, acetyl-CoA carboxylase, fatty acid synthase, and malic enzyme are regulated by changes in the transcriptional rate, G6PD is regulated solely by posttranscriptional mechanisms [1,2]. Posttranscriptional regulation has been proposed for malic enzyme and fatty acid synthase [3–5], as well, indicating that nutrients regulate via multiple mechanism even for the same gene.

Posttranscriptional regulation of mRNA abundance can occur at multiple steps during RNA processing or through changes in the stability of the mature mRNA. The processing of a nascent transcript includes addition of the 7-methylguanosine cap, 3'-end formation, and splicing of all introns. Accurate processing is essential for the release of the mRNA from its transcription site and for export of the mRNA to the cytoplasm [6]. Thus, efficient and complete maturation of mRNA is a potential control point in gene expression. Splicing of mRNA requires distinguishing exons from introns. Within introns several sequences bind components of the spliceosome; these include the 5' and 3' splice site, the branch point, and the polypyrimidine tract [7]. Additional sequences in both the exons and introns function to regulate the efficiency of splicing. Within exons, the splicing regulatory sequences are referred to exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) and these sequences affect the recruitment of components of the spliceosome [7–9]. Introns contain analogous regulatory sequences and these function to either enhance or block splice site recognition [10]. Families of serine-arginine rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs) bind to these regulatory sequences. In general, SR proteins bind ESEs and enhance splicing, while ESSs or their intronic counterparts are bound by hnRNPs and splicing is silenced [8,11–14]. Examples also exist of SR proteins inhibiting splicing and hnRNPs enhancing splicing [15, 16]. Thus considerable research remains in order to understand how cis-acting RNA elements and their binding proteins regulate splicing.

We characterized the posttranscriptional regulation of G6PD by dietary factors. Refeeding mice after starvation causes a 12- to 15-fold increase in G6PD mRNA abundance in the liver, while addition of polyunsaturated fatty acids to the diet or to the medium of primary rat hepatocytes caused an 80% decrease in G6PD mRNA [17]. Despite this large change in mRNA accumulation, the rate of transcription of the G6PD gene does not change. Rather, the changes in G6PD mRNA abundance during refeeding of starved mice or by fatty acids are caused by an in the rate of splicing of the primary transcript [18]. In this regard, changes in the accumulation of the mature mRNA are preceded by changes in the rate of accumulation of partially spliced mRNA in the absence of changes in polyadenylation or length of the poly (A) tail. If splicing is the regulated step, then sequences within G6PD RNA should be required for this regulation. This hypothesis was tested by transfection of RNA reporter constructs into primary rat hepatocytes. These constructs contained portions of the G6PD gene encoding the pre-mRNA ligated to the CMV promoter, which is not regulated by nutrients. Expression of RNA from this reporter was inhibited by treatment of the hepatocytes with fatty acids. Using deletion mutagenesis of the reporter DNA a cis-acting element required for the control of G6PD mRNA accumulation was localized to exon 12 of the G6PD pre-mRNA. Inclusion of exon 12 and its surrounding introns was required for the inhibition of reporter RNA expression by polyunsaturated fatty acids. Furthermore, this exon when ligated to a heterologous RNA confers regulation by nutrients to this RNA [19]. These characteristics are consistent with exon 12 containing an enhancer or a silencer element. Unique to this regulation is that it is the rate of exon splicing that is regulated; G6PD mRNA is not alternatively spliced to form different isoforms.

Constitutive exons are those always present in the mature mRNA. The regulated splicing of G6PD mRNA involves a constitutive exon in this mRNA and a decrease in splicing of this exon diminishes expression of this mRNA. This is a primary mechanism by which G6PD

expression is regulated [2]. Molecular mechanisms involved in the exclusion and inclusion of alternatively spliced exons have been characterized. Little is known about the mechanism regulating the rate of constitutive exon splicing particularly in response to hormonal or nutritional factors. The proteins involved in regulation of inclusion of alternatively spliced exons may also be involved in the splicing of constitutive exons particularly those with weak splice sites. To understand the molecular mechanisms involved in regulation of G6PD splicing, we sought to identify the proteins that bind to exon 12 of G6PD mRNA. The large change in the accumulation of G6PD mRNA during the starvation to refeeding transition makes this dietary paradigm ideal for the identification of regulatory proteins particularly those whose binding might change in response to nutritional status. In this report, we present evidence that hnRNPs K, L and A2/B1 bind to the exon 12 splicing regulatory element. A region of G6PD exon 12 from nt 65–79 is sufficient to bind these proteins. The binding of these proteins is enhanced by starvation and correlates with the decrease in G6PD mRNA abundance in the livers of starved mice. These data are consistent with a role for these proteins silencing the splicing of G6PD pre-mRNA. The identification of these regulatory proteins provides new insight into the mechanisms by which nutrients control gene expression.

2. Materials and Methods

2.1. Animal Care

Four to six week old, male, C57BL/6 mice (Hill Top) were maintained on a high-carbohydrate diet with glucose as the carbohydrate source (Purina Mill) and supplemented with 1% (by weight) safflower oil (Sigma) as a source of essential fatty acids for seven days. On day eight, the food was removed for a 24 h starvation period. At the end of the starvation period, 10 mice were sacrificed and 10 were refed the high-carbohydrate diet for 24 h.

2.2. Plasmids and In vitro Transcription of RNA

The plasmid containing exon 12 of G6PD mRNA was generated by PCR using the 5' primer AATAAGCTTTGATGAACTCAGGGA and the 3' primer ATTTCTAGACTGCCATATACATAG. The exon 11–13 plasmid was generated by subcloning from a genomic clone following restriction digestion using PstI and KpnI. Exon12 Δ^1 probe (nt 37–93 of exon 12) was generated using PCR with the 5' primer ATAAAGCTTCTGCTGCACAAGATTGAT and the 3' primer above. Exon12 Δ^2 probe (nt 47–93 of exon 12) was generated with the same 3' primer and the 5' primer TGAAAGCTTATTGATCGAGAAAAGCC. All DNA inserts were subcloned into pKS+ plasmid using Hind III and Xba I sites created with the primers (Fig. 1A). RNA probes were made using an in vitro transcription reaction with T3 RNA polymerase (Ambion) as previously described [20].

2.3. RNA Oligonucleotides

RNA oligonucleotides corresponding to 15 or 30 nt regions of exon 12 (Fig. 1B) were purchased from IDT (Coralville, IA). An RNA oligonucleotide, UAGGGACUUAGGGUG (positions 6 to 20nt of a consensus sequence for hnRNP A1) was used as a positive control [21]. As a negative control, an oligonucleotide, CAAAAGCAUGCAAAA, was designed to lack known RNA regulatory elements as screened by the ESE and ESS finder databases: <http://exon.cshl.edu/ESE> [22], <http://genes.mit.edu/fas-ess> [23], and <http://cubweb.biology.columbia.edu/pesx> [24]. RNA oligonucleotides were end-labeled with (γ -P³²) ATP using a kinase Max kit (Ambion).

2.4. UV Crosslinking

Nuclear extracts were prepared by the Dignam protocol [25]. Protein concentration was measured by the Bradford assay. UV crosslinking reactions contained 5 to 10 µg of nuclear extract protein, 10 to 20 fmol of substrate RNA (50,000 to 100,000 cpm), 1 mM ATP, 0.7 mM MgCl₂, and 40 ng of carrier tRNA in a 25 µl final volume of buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.9, 0.2 mM EDTA, 10% glycerol, and 150 mM KCl) [26]. Reaction mixtures were incubated for 5 to 10 min at 30°C and then subjected to UV crosslinking on ice for 10 min in a Stratalinker (1.8 x 10⁶ µJ/cm²). Samples were treated with (1 mg/ml) at 37°C for 30 min. Proteins were boiled for 5 min in cracking buffer (80 mM Tris-Cl, pH 6.8, 0.1 M dithiothreitol, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.2% bromophenol blue) and separated in 10% SDS-polyacrylamide gels [26]. Binding of proteins to the RNA was visualized using storage phosphor imaging and quantified using ImageQuaNT software.

2.5. RNA Affinity Purification of Binding Proteins

RNAs were covalently linked to adipic acid dihydrazide-agarose beads by a modification of a published procedure [27,28]. Briefly, 1000 pmol of RNA were placed in a 400 µl reaction mixture (100 mM sodium acetate, pH 5.0, and 5 mM sodium m-periodate) and incubated for 1 h in the dark at room temperature. Following ethanol-precipitation, the RNA was resuspended in 500 µl of 0.1 M sodium acetate, pH 5.0. Adipic acid dihydrazide-agarose beads (400 µl) (Sigma) were washed four times in 10 ml of 0.1 M sodium acetate, pH 5.0, resuspended in 1 ml of 0.1 M sodium acetate, pH 5.0 and mixed with the periodate-treated RNA by rotation for 12 h at 4°C. The beads with the bound RNA were washed three times in 2 M NaCl and three times in 20 mM HEPES-KOH, pH 7.6, 10% v/v glycerol, 150 mM KCl, 0.2 mM EDTA and 200 µg/ml tRNA to block nonspecific protein binding to the beads. The beads containing immobilized RNA were incubated with mouse liver nuclear extracts (100–500 µg protein) in 300 µl of 20 mM HEPES-KOH, pH 7.6, 10% v/v glycerol, 150 mM KCl, 0.2 mM EDTA plus 2.5 mM ATP, 2.0 mM MgCl₂, 1000 ng/ml tRNA for 30 min at 30°C. Beads were pelleted by centrifugation and washed four times with 1 ml of buffer without ATP, MgCl₂ or tRNA. For one dimensional gels and Western analysis, the protein were eluted in 75 µl of 80 mM Tris-Cl, pH 6.8, 0.1 M dithiothreitol, 2% SDS, 10% glycerol, and 0.2% bromophenol blue by heating for 5 min at 95 °C. For two-dimensional gel electrophoresis, the proteins were eluted in 250 µl of 8M Urea, 2% Triton X-100, 1% dithiothreitol, 0.5% Pharmalyte, and 0.002% bromophenol blue for 2–3 h at room temperature.

2.6. Two-Dimensional Gel Electrophoresis

Proteins eluted from the beads were separated by isoelectric focusing (13 cm strips; Amersham) with a pH range of 3.0 to 10.0 in the first dimension. The gels were hydrated by incubation with the elution buffer containing the purified proteins for 12 h followed by separation at 50 volts for 5 h, 100 volts for 2 h, 1000 volts for 2 h, 5000 volts for 2 h, 8000 volts for 6 h and maintained at 1000 volts for 4 h. The gels were then incubated in equilibration buffer (50 mM Tris-Cl, pH 6.8, 6 M Urea, 20% glycerol, 2% SDS, 0.002% bromophenol blue) plus 15 mg/ml of dithiothreitol for 15 min plus an additional 15 min in equilibration buffer plus iodoacetamide (18 mg/ml). The gels were placed horizontally onto an SDS–10% polyacrylamide gel and run at 100 V for 6–8 h. For quantitation of the proteins, the gels were stained with SYPRO Ruby using the manufacture's protocol and visualized using an excitation of 450 nm and emission of 610 nm on a Typhoon Scanner (GE Healthcare). For visualization of proteins prior to excision of protein spots, the gels were stained with colloidal Coomassie blue (Invitrogen) and visualized under a light box. The visualized spots were excised from the gel and stored at –80° C.

2.7. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

Proteins binding to the RNA coated beads were separated by size in a 10% SDS-polyacrylamide gel. The proteins bands of interest were visualized by colloidal Coomassie blue and excised from the gels. The gel slices were digested with trypsin (2 µg/ml) overnight at 37°C. The digested peptides were dried and reconstituted in 5% acetonitrile, 0.1% formic acid and then loaded onto a C₁₈ column using a helium pressure cell. Peptides were eluted from the column using a linear acetonitrile gradient of 5–50% over 60 min with a flow rate 300 nl/min. The ion-trap mass spectrometer (ThermoFinnigan LCQ Deca Plus) was programmed to perform a full MS scan followed by MS/MS scans of the five most abundant ions present. Raw data files were compared to the Swiss-Prot database using SEQUEST software to identify proteins that match the sequence of the peptide fragments. The criteria we used to determine the protein identity were a ΔC_n score of less than 0.1, regardless of the charge state and a cross correlation (X_{corr}) score of at least 1.9, 2.2, or 3.7 for the +1, +2, or +3 charge states, respectively [29]. In a few cases, peptides with an ΔC_n score of greater than 0.1 were accepted if the X_{corr} was strong. Protein identifications were manually confirmed [30].

2.8. Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry Analysis

Following separation in the two-dimensional gels, the colloidal Coomassie blue stained gel pieces containing proteins were destained using a 1:1 ratio of 100 mM ammonium bicarbonate and methanol for 30 min followed by 30 min with 100 mM ammonium bicarbonate and acetonitrile for 10 min. The samples were dried under a vacuum and rehydrated overnight in 2 µg/ml of trypsin in 25 mM ammonium bicarbonate at 37°C. The samples were sonicated for 15 min in 2 volumes of acetonitrile. The peptides were purified using a ZipTip C₁₈ (Millipore) pre-equilibrated with 10% acetonitrile and 0.1% trifluoroacetic acid. The samples were eluted from the ZipTip using 2% acetic acid and 50% acetonitrile solution. The resulting sample was mixed with matrix (50 mM α -cyano-cinnamic acid in 49.5% ethanol, 49.5% acetonitrile and 0.0001% trifluoroacetic acid solution. The MALDI 96 well plate was spotted with 1 µl in each well and air-dried. The MALDI-TOF (Proteomics Workflow) data was evaluated using the MASCOT software.

2.9. Western Blot Analysis

Eluted proteins from the adipic acid dihydrazide-agarose beads bound to RNA oligonucleotides were separated on 10% SDS-polyacrylamide gel. Western analysis was as previously described [31]. The primary antibodies against hnRNP K, L and A2/B1 were obtained from ImmuQuest. Anti-mouse IgG2b (Zymed) and IgG (Bio-Rad) conjugated to horseradish peroxidase were used as secondary antibodies to detect hnRNP K, and L and A2/B1, respectively. The immunocomplexes were detected by enhanced chemiluminescence (Pierce). Images were visualized with film (Pierce) and quantified by densitometry using ImageQuant (Molecular Dynamics).

3. Results

3.1. Starvation enhances binding of proteins to exon 12 RNA

Regulation of G6PD expression both in mouse liver and primary rat hepatocytes occurs by changes in the rate of pre-mRNA splicing [18,32]. Exon 12 contains a regulatory element involved in regulated splicing of G6PD pre-mRNA [19]. Therefore, we used exon 12 as a probe (Fig. 1A) in UV crosslinking assays with mouse liver nuclear extracts to identify RNA binding proteins that may be involved in regulated splicing. Multiple proteins bound to exon 12 RNA. Unique bands were consistently observed at approximately 60 kDa, 50 kDa and 37 kDa (Fig. 2A, lane 1). Additional protein bands at 75 kDa and 150 kDa were not observed in all

experiments, and a 100 kDa band was observed with multiple RNA probes including the transcribed multiple cloning site of pKS+ (data not shown). Thus, the 75, 100 and 150 kDa bands were interpreted to be non-specific binding caused by UV crosslinking and such band are not atypical of this assay (cf., Fig. 2A versus Fig. 5, first lane each panel). Multiple controls were used to verify that the observed bands represented protein bound to the RNA (data not shown). Competition experiments with unlabelled exon 12 RNA probe were performed. The bands at 60 kDa, 50 kDa, and 37 kDa were decreased by unlabelled RNA probe in a concentration dependent manner. UV crosslinking of the RNA probe in the absence of nuclear extract did not result in any bands. Furthermore, digestion of the RNA/protein complex with proteinase K eliminated all bands verifying that they are bound protein and not double stranded RNA molecules. Thus, the pattern of bands observed in this assay represents proteins binding to the RNA probe.

The amount of protein binding to exon 12 varied with nutritional status of the mice. Refeeding with a high-carbohydrate diet resulted in a decrease in the intensities of the protein bands at 60 and 37 kDa. These bands designated as A and B, respectively, were decreased by almost 50% (Fig. 2A compare lanes 1 and 5), and this decrease in protein binding exceeded the minor differences in intensity observed with the non-specific band at 100 kDa. Furthermore, the inhibition of binding was consistent across multiple nuclear extract preparations (Fig. 2B). These nuclear extracts were prepared from intact livers. During refeeding, the amount of glycogen in liver increases dramatically (25% increase in liver weight, [33]); glycogen can contaminant cellular fractions. A remote possibility is that these differences in band intensity reflect differences in the concentration of glycogen in the nuclear extract that artificially affected protein binding. Purified glycogen (1 to 5 μ g) was added to the UV crosslinking reactions using the starved nuclear extracts. The pattern and intensity of protein binding was not altered by the addition of glycogen (data not shown). In addition, silver staining of nuclear extracts separated on polyacrylamide gels further confirmed that overall protein content was similar between starved and refeed extracts (data not shown). Thus these differences in protein binding between dietary states are consistent with the idea that these proteins at 60 and 37 kDa are involved in the regulated splicing of G6PD mRNA.

We next asked if additional differences in protein binding or intensity would be observed if we included the introns surrounding exon 12 in the RNA probe. Using a construct containing exon 11 through 13 with surrounding introns, a similar pattern of protein bands was observed as with exon 12 alone (Fig. 2A, lanes 2 and 6). A band at 55 kDa was unique to the exon 11–13 RNA probe. Importantly, the decrease in protein binding at bands A and B during refeeding was retained with this longer construct (Fig. 2B). The intensity of the band at 55 kDa did not vary across multiple experiments. Thus, starvation and refeeding cause major differences in the binding of proteins to an exon involved in regulated splicing of G6PD pre-mRNA.

3.2. The 60 kDa and 37 kDa proteins bind to nt 65–79 of exon 12

To further localize the binding sites for the 60 kDa and 37 kDa proteins in bands A and B, two RNA probes containing 5' deletions of exon 12 sequences were synthesized. These probes had deletions of the first 37 and 47 nt from exon 12, respectively (Exon 12 Δ^1 and Exon 12 Δ^2 ; Fig. 1A). The pattern of protein binding to these probes was the same as to the full-length exon 12 (Fig. 2A, lanes 3 and 4). In addition, the decrease in binding at bands A and B during refeeding was maintained in these shorter RNA probes (Fig. 2A, lanes 3 and 4 versus 7 and 8, and Fig. 2B). The first 37 nt of exon 12 are not required for regulated splicing of G6PD mRNA in rat hepatocytes [19].

An RNA oligonucleotide approach was used to further localize the region of exon 12 that binds the 60 and 37 kDa proteins. RNA oligonucleotides, 15 nt in length were synthesized across sequence from 35–93 of exon 12 and were used in the UV crosslinking assay (Fig. 1B). An

oligonucleotide from 35–49 that overlaps with the 47 nt deletion above, functioned as a negative control. Unique proteins bound to each RNA oligonucleotide (Fig. 3). Bands A and B associated with full-length exon 12 were only observed with the oligonucleotide, nt 65–79 (Fig. 3). The intensity of bands A and B decreased in the nuclear extracts from livers of refed mice, similar to the decrease observed with the full-length exon 12 RNA probe. Apparent differences in protein binding observed with the other oligonucleotides (bands 1–6) were not consistently observed in five separate crosslinking assays and with different preparations of nuclear extracts. A positive control was designed to detect overall differences in protein amounts between starved and refed nuclear extracts. Burd and Dreyfuss identified a 20 nt sequence that is bound by hnRNP A1 as well as other members of the hnRNP family [21]. The hnRNP A1 oligonucleotide was designed to contain nucleotides 6–20 of the Burd and Dreyfuss sequence including the consensus UAGGGA sequence for hnRNP A1 binding [21]. In the UV crosslinking assay, multiple bands were detected with the hnRNP A1 oligonucleotide including an intense doublet at 37 kDa, corresponding in size to hnRNP A1 (Fig. 3). The intensity of the bands did not differ between starved and refed nuclear extracts (n=12 nuclear extracts; data not shown). An additional control was a non-specific oligonucleotide representing a random RNA sequence that did not contain known binding sites for splicing regulatory proteins, as determined using the databases indicated in Material and Methods. Binding of proteins to this oligonucleotide was not detected (Fig. 3). These data further verify that the differences in protein binding between nuclear extracts from starved and refed mice are due to differences in the interaction of the 60 kDa and 37 kDa proteins with nt 65–79.

3.3. RNA affinity purification of proteins binding to nt 65–79 of exon 12

To determine the identity of the proteins in bands A and B, RNA oligonucleotides were linked to adipic acid beads and used to purify proteins from the nuclear extract without covalently linking them to the RNA. We first asked if this assay resulted in a similar pattern of protein binding as the UV crosslinking assay. Proteins binding in the 60 and 37 kDa regions (bands A and B, respectively) were detected with nt 65–79 (Fig. 4). The 60 kDa band was not detected with nt 35–49, nt 50–64, nt 70–93 or the non-specific oligonucleotide. The 37 kDa band was detected with nt 50–64, but not with nt 35–49, nt 79–93 or the non-specific oligonucleotide. Proteins were not detected co-purifying with the beads alone (Fig. 4). As observed with the UV crosslinking assay, the amount of proteins bound in bands A and B were 60% and 53% less, respectively in the nuclear extracts from refed mice. Thus, the assay recapitulated the differences in protein binding at bands A and B observed with UV crosslinking.

Steric hindrance between the short RNA oligonucleotides and the large beads could have decreased overall protein binding. To facilitate maximum protein pull-down for identification of the proteins with LC-MS/MS, a larger oligonucleotide spanning nt 50–79 of exon 12 was used in the RNA affinity assay (Fig. 4). An identical pattern of proteins was observed with this oligonucleotide as with nt 65–79; however, more protein per band bound to nt 50–79 and the inhibition of protein binding in nuclear extracts from refed mice (51% and 44% for bands A and B, respectively) was maintained. The proteins bound to exon 12 were the same size as the proteins bound to nt 50–79. To test if the bound proteins were the same, competition analysis was used. The RNA oligonucleotide, nt 50–79 was used as a competitor in the UV crosslinking assay with the full-length exon 12 as the probe (Fig. 5). Increasing amounts of unlabeled nt 50–79 were added to the reaction. This oligonucleotide effectively competed with the labeled exon 12 probe for binding of the proteins in bands of A and B (Fig. 5, lane 1 versus lanes 2–4). In contrast, the non-specific oligonucleotide was unable to compete with exon 12 for these proteins (Fig. 5, lane 1 versus lanes 5–7). Thus, the region from nt 50–79 of exon 12 accounts for the binding of the 60 kDa and 37 kDa proteins to the full-length exon.

3.4. HnRNPs K, L and A2/B1 bind to exon 12, and the binding is regulated by nutritional status

The RNA affinity assay was used to purify the proteins in band A and band B in order to determine their identity using LC-MS/MS. Only starved nuclear extract was used and the reaction was increased five-fold to provide sufficient protein for the LC-MS/MS analysis. Despite the increase in protein, the specificity of binding of the 60 kDa and 37 kDa proteins (bands A and B) to nt 65–79 and nt 50–79 was retained (Fig. 6, lanes 3 and 6). Bands A and B were excised from the gel and an in-gel digestion with trypsin was performed prior to LC-MS/MS analysis (Fig. 6). Band A contained two proteins, hnRNP K and hnRNP L. The percent sequence coverage of hnRNP K was 35.7% and 14 unique peptides were sequenced. The coverage for hnRNP L was 33.2% with 16 unique peptides (Table 1). Band B contained hnRNP A2/B1, which was identified based on sequence coverage of greater than 60% and with 21 unique peptides (Table 1). HnRNPs A2 and B1 are isoforms generated by alternative splicing and differ by 12 amino acids that are present in exon 2 of hnRNP B1 but not in hnRNP A2 [34]. A manual search for a peptide containing this amino acid sequence yielded the peptide, TLETVPPL, with an X_{corr} of 2.9 for a +2 charge-state peptide and a ΔC_n value of greater than 0.5. This peptide was from amino acids 4–12 of exon 2 and confirmed that hnRNP B1 is in band B. Because both proteins share all other peptides, the occurrence of a peptide unique to hnRNP B1 does not exclude hnRNP A2 from also being present in band B. The molecular weight of the three identified proteins matched their mass predicted from the SDS-polyacrylamide gels. HnRNP C1/C2 was also identified in band B. Seven peptides of this protein were sequenced and the percent sequence coverage was less than 20% making identification of this protein inconclusive.

To verify these protein identifications and to determine if additional proteins bound to nt 50–79, the proteins eluted from the adipic-acid beads were resolved in two-dimensional gels and identification of protein spots was performed by MALDI-TOF. Once again, the hnRNP proteins K, L, and A2/B1 were detected bound to this sequence. Multiple spots corresponding to hnRNP A2/B1 were observed. HnRNP A2 migrates at a more acid pH in 2 dimensional gels [35]. Thus, this is consistent with both isoforms binding to the RNA element but the existence of one isoforms versus the other cannot be determined by MALDI-TOF analysis. The identifications of hnRNPs K, L and A2/B1 were based on sequence coverage of 45%, 24% and 55%, respectively. HnRNP C1/C2 was not detected in the two-dimensional analysis. Three additional proteins, dematin, p47 and SAP 114 were also detected bound to nt 50–79 (Fig. 7; asterisk). These proteins are not directly involved in regulation of RNA splicing. They may have bound non-specifically to one of the proteins pulled-down by the RNA-linked beads, and thus, their intensity was decreased in the refed samples because the amount of their interacting partner, possibly hnRNP K was also decreased. Additional proteins in the 2D gels were not sequenced because their concentration was too low to permit positive protein identification. Proteins of greater intensity in the refed nuclear extracts were not observed consistent with the previous data using UV crosslinking and one-dimensional gel analysis after affinity purification.

The identification of hnRNPs K, L, and A2/B1 was confirmed using Western blot analysis. Antibodies against hnRNPs K and L detected bands at 65 kDa binding to oligonucleotides 65–79 and 50–79 (Fig. 8). Although the predicted molecular mass of hnRNP K is 50.9 kDa, it migrates in polyacrylamide gels at 65 kDa ([36] and Immunoquest product literature). Little or no protein bound to either the beads alone, to the non-specific oligonucleotide (Fig. 8), or to the oligonucleotide, nt 79–93 (data not shown). Furthermore, refeeding decreased the binding of hnRNPs K and L to nt 50–79 by $70 \pm 8\%$ and $62 \pm 3\%$, respectively, for three separate pull-down assays. HnRNP A2/B1 was detected as a broad band at 37 kDa. The antibody detects both isoforms of this protein. The amount of hnRNP A2/B1 bound was decreased by 70.1% ($n=2$ pull-down assays) in extracts from refed mice. The amount of these

proteins in the total nuclear extracts (Fig. 8, input) did not vary with nutritional status. Thus, nutritional status regulates the binding of hnRNP K, L, and A2/B1 to a region of exon 12 from nt 65–79 in the absence of a change in their nuclear concentration.

4. Discussion

Over half of all genes undergo alternative splicing either in response to hormonal, developmental signals or in a tissue-specific manner. This process is highly regulated. An increasing number of genes have been identified whose expression is regulated by changes in the rate of splicing of constitutive exons; G6PD is one such gene [2,37–39]. Unique to the regulated splicing of G6PD is that it occurs in response to nutritional cues [2]. In this regard, the 12- to 15-fold increase in G6PD mRNA accumulation during the starvation to refeeding transition is caused by comparable changes in the rate of RNA splicing [18]. This large change in mRNA accumulation makes this an excellent dietary paradigm in which to study the molecular details involved in regulated splicing. Our work has identified a cis-acting regulatory element in exon 12 of the G6PD mRNA that is required for regulated splicing of this RNA [19]. In this report, we present evidence that hnRNP K, L, and A2/B1 differentially bind to exon 12 in response to starvation and refeeding.

The inverse correlation between increased binding of hnRNP K, L and A2/B1 to G6PD mRNA and a decrease in the abundance of G6PD mRNA during starvation suggests that these proteins function to inhibit G6PD splicing. Only a few regulatory proteins have been described that function to inhibit RNA splicing and among these are hnRNP L and A2/B1. HnRNP L binds to an ESS in the CD45 mRNA and silences inclusion of three alternatively spliced exons within this RNA [14,40]. In each alternatively spliced exon of CD45 pre-mRNA, hnRNP L functions within a composite RNA regulatory element that also contains an ESE and a regulatory sequence that facilitates splicing regulation in response to external stimuli. In contrast to this splicing silencing activity of hnRNP L, this protein also functions to enhance splicing of the nitric oxide synthase pre-mRNA [15]. HnRNPs of the A and B group function in the inhibition of *tat* exon 1 splicing in the HIV-1 pre-mRNA [27,41]. The different isoforms within this group are equally effective in causing this inhibition. Because hnRNP A2 and B1 cross-reacted with the antibody used in the Western analysis (Fig. 8), the presence of both hnRNPs or just hnRNP B1 cannot be determined. None-the-less, the binding of this group of hnRNPs to G6PD exon 12 is consistent with it functioning in the inhibition of G6PD expression during starvation. A potential role for hnRNP K in inhibiting G6PD splicing is less clear. HnRNP K has ubiquitous functions in RNA metabolism; its roles in RNA translation and mRNA stability are the best-characterized [42]. The ability of hnRNP K to interact with other proteins involved in RNA processing [43] suggests that it may have a permissive function, recruiting proteins that directly interact with components of the spliceosome.

Sequence specific details by which these proteins bind to their RNA elements are not clear. The sequence between nt 65 and 79 of exon 12 is the minimal sequence required for binding of these proteins, while binding is enhanced by inclusion of the upstream 15 nt. HnRNP L binds to CA repeats in the nitric oxide synthetase mRNA but this binding enhances mRNA splicing [15]. HnRNP L binds to an ESS in the CD45 mRNA [14]. The G6PD regulatory element does not resemble this sequence and neither of these elements scores highly using software designed to search for splicing silencing elements. HnRNPs of the A/B family are predicted to bind tandem UAG repeats [21,44]. This sequence is not present in the G6PD regulatory element. The G6PD regulatory sequence does score highly as a potential exonic regulatory sequence using a new algorithm developed using computational analysis of 46,103 exons [45]. This is consistent with our most recent data demonstrating that the region from nt 43–72 of exon 12 is an ESS [46].

The nt 65–79 sequence in exon 12 of G6PD mRNA does contain two C-rich stretches, predictive of hnRNP K binding sites [42]. Mutation of the three C's from nt 65–67 markedly decreases the binding of all proteins to the RNA (Griffith, B.N. and Salati, L.M., unpublished data) and corroborates the finding that hnRNP K binds to this element. hnRNP K interacts with a large number of nuclear proteins involved in splicing including hnRNPs L and A2/B1 [43]. The observation that elimination of a potential hnRNP K binding site also decreased the binding of hnRNPs L and A2/B1 is consistent with the idea that the binding of hnRNP K facilitates the interaction of hnRNPs L and A2/B1. Because these in vitro assays eliminate regulatory functions of nuclear structure on RNA/protein interactions, conclusions regarding their role in regulated splicing cannot be made. The physiological relevance of the interaction of these hnRNPs with the G6PD regulatory element must be tested directly; these experiments are on-going in the laboratory.

Regulatory elements found within exons can act as splicing enhancers or silencers depending on the proteins that bind these sequences. Most enhancers bind members of the SR family of proteins, while silencers bind members of the hnRNP family [8,11–14]. In general, the binding of SR proteins to ESEs enhances the recruitment of spliceosome components to the exon 12. SR protein binding might have been expected using nuclear extracts from refed mice. Bands increasing in intensity were not observed nor were these proteins detected in the 2-dimensional gels. Thus, these proteins either do not bind within this sequence or the amount bound was below the limits of detection. Because hnRNPs L and A2/B1 are known to bind ESSs and act as splicing silencers, the binding of these proteins to G6PD pre-mRNA is consistent with this element being an ESS [46].

Three additional proteins were also identified by MALDI-TOF analysis. Dematin is a known actin filament bundling protein that is not known to play a role in splicing [47]. The protein, p47 is similar to a zinc finger protein (Zfp462), but the function of this protein is not known (Swissprot database). SAP 114 protein, is a subunit of the spliceosome referred to as the spliceosome associated protein, but the molecular weight of this protein is 30 kDa and does not match the 60 kDa molecular weight observed in the gel and perhaps co-purified with another protein [48]. Therefore, the significance of these three proteins is not clear at this point.

The changes in binding of hnRNPs K, L and A2/B1 to exon 12 occurred in the absence of changes in the total amount of these proteins in the nuclear extracts (Fig. 8). hnRNPs are known to undergo posttranslational modifications such as phosphorylation [49,50], methylation [51, 52], and sumoylation [53]. In the case of hnRNP K, phosphorylation can regulate RNA binding [49]. It is likely that posttranslational modifications control the binding of hnRNP K, L, and A2/B1 to the RNA regulatory element within G6PD mRNA. Phosphorylation can cause a shift in the mobility of proteins in two-dimensional gels [54]. A shift was not observed suggesting that this is not the case. Determining the mechanism for these changes in protein binding and how they regulate mRNA splicing is the subject of on-going research in our laboratory. Determination of how hnRNP K, L and A2/B1 differentially bind to the RNA regulatory element within exon 12 will be fundamental in the elucidation of novel regulation mechanisms involved in the nutritional regulation of gene expression.

Acknowledgements

*We thank Dr. Timothy Vincent and the proteomics facility for protein identifications and for helpful discussions, and Dr. Massimo Caputi for advice with the adipic acid bead protocol. We acknowledge the contribution of Ms. Xiaohui Hou for constructing the exon 11–13 probe. This work was supported by National Institutes of Health Grant DK46897 (to L.M.S.), by a pre-doctoral fellowship from the American Heart Association Mid 0315129B (to B.N.G), and by the COBRE for Signal Transduction and Cancer (5P20RR016440-05) and the WVU Proteomics Facility

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Fig 1.

The structure of the G6PD RNA probes used in the identification of RNA binding proteins. (A) The G6PD gene contains 13 exons and 12 introns of which exon 12 is known to regulate splicing in primary hepatocytes [19]. The exon 12 probe contained the entire exon 12 sequence. The Exon 11–13 probe included exons 11 and part of exon 13 and the surrounding introns. The Exon 12 Δ^1 and Δ^2 probes had 5' deletions of 37 and 47 nucleotides from the exon, respectively. (B) The sequence from nt 35–93 is diagrammed. The lines beneath the sequence indicate the sequence of 15 or 30 nt RNA oligonucleotides used in the UV crosslinking and RNA affinity assays. An oligonucleotide (oligo) containing an hnRNP A1 binding site was used as a positive control in these assays. The non-specific oligonucleotide represents a random sequence of nucleotides predicted not to bind to RNA binding proteins.

B

	A	B
	% Decrease	
Exon 12	49.9 ± 4	46.3 ± 9
Exon 11-13	31.4 ± 5	51.0 ± 6
Exon 12 Δ^1	45.5 ± 8	48.0 ± 15
Exon 12 Δ^2	58.5 ± 6	56.9 ± 12

Fig 2.

Detection of RNA binding proteins by UV crosslinking analysis. (A) Mice were starved for 24 hrs and then half were refed a high-carbohydrate diet for an additional 24 hrs; liver nuclear extracts were prepared and UV crosslinked to the exon 12 RNA probes diagrammed in Fig. 1A. The bands indicated by asterisks indicate non-specific protein binding. A and B refer to bands of 60 kDa and 37 kDa, respectively, that changed in intensity between starved and refed mice. (B) The protein bands A and B were quantified using ImageQuant software. The percent-decrease was calculated by dividing the phosphor imager units from starved by the value from refed samples. The values represent the mean plus or minus the standard error of six different nuclear extracts.

Fig 3.

The 60 kDa and 37 kDa proteins regulated by starvation and refeeding bind to nt 65–79 of exon 12. Mouse liver nuclear extracts from the livers of mice that were starved (S) or refed (R) were UV crosslinked to end-labeled RNA oligonucleotides (oligo) to the indicated regions of exon 12 or to the uniformly labeled full-length exon 12 probe (Exon 12). The hnRNP A1 consensus sequence was included as a positive control. The non-specific (NS) oligonucleotide is an RNA oligonucleotide that was designed to lack known sites for splicing factor binding. A and B refer to the 60 kDa and the 37 kDa bands, respectively. The numbers 1–6 represent protein bands unique to each oligonucleotide.

Fig 4.

RNA affinity assay to purify RNA binding proteins. RNA oligonucleotides (oligo) were covalently linked to adipic acid beads and used to pull-down RNA binding proteins. The region of exon 12 represented by each oligonucleotide is indicated under the gel. The RNA bound beads were incubated with nuclear extracts (100 μ g of protein) from the livers of starved (S) and refed (R) mice. The eluted proteins were separated by size on an SDS polyacrylamide gel and visualized using Sypro Ruby. A and B refer to the 60 kDa and 37 kDa proteins. Beads alone were the adipic acid beads without linked RNA. The non-specific (NS) oligonucleotide was a negative control.

Fig 5.

The 60 kDa and 37 kDa proteins binding to full-length exon 12 and nt 50–79 are the same. The uniformly labeled exon 12 probe was incubated with mouse liver nuclear extract from starved mice in the presence of 0, 100, 1000, and 10,000-fold Molar excess of competitor. The first competitor was unlabeled nt 50–79, and the second competitor was the non-specific (NS) RNA oligonucleotide.

Fig 6.

RNA affinity assay for LC-MS/MS identification of the proteins within bands A and B. Adipic acid beads bound to the indicated RNA oligonucleotides were incubated with nuclear extract (500 μ g of protein) from starved mouse livers. The boxes represent the regions that were excised from the gel prior to trypsin digestion. The input lane was 10% of the amount of protein used in the RNA affinity assay. NS was the non-specific oligonucleotide.

Fig 7.

Confirmation of protein identities by two-dimensional gel electrophoresis and MALDI-TOF analysis. Liver nuclear extracts (500 µg of protein) from starved and refed mice were incubated with adipic acid beads linked to the RNA oligonucleotide, nt 50–79. The purified proteins were eluted from the beads and analyzed by isoelectric focusing in a pH gradient from 3 to 10 followed by size separation in 10% polyacrylamide. The image shown is a Sypro Ruby stain of protein in the gel. Each spot was excised from the gel and protein identity was determined using MALDI-TOF analysis and MASCOT software. The asterisks are p47, dematin, and SAP 114.

Fig 8.

HnRNP L, K and A2/B1 differentially bind to the exon 12 RNA regulatory element in starved versus refed mice. RNA oligonucleotides to nt 65–79, nt 50–79 of exon 12, or the non-specific (NS) oligonucleotide were covalently linked to adipic acid beads and incubated with mouse liver nuclear extracts (100 µg of protein) from starved (S) and refed (R) mice. The proteins were eluted from the beads and 2 µl of eluted proteins were analyzed by Western analysis using the indicated antibodies (IB). Beads alone were adipic acid beads without a linked RNA oligonucleotide. The input lane was 10% of the amount of nuclear extract used the RNA affinity assay.

Table 1

Assigned identities of proteins bound to nt 50–79 of exon 12. RNA affinity purified proteins were separated by size and the bands representing regions A (60 kDa) and B (37 kDa) were excised from the gel as described in Fig. 6. LC-MS/MS was performed as described in Materials and Methods. The peptide sequences were compared to the mouse protein database.

	Protein	Accession #	Mass (kDa)	Sequence coverage	Unique peptides
Band A	hnRNP L	796275	60.4	33.2%	16
	hnRNP K	079555	50.9	35.7%	14
Band B	hnRNP A2/B1	872591	37.4	60.1%	21