

# Genome-wide Analysis of Alternative Pre-mRNA Splicing and RNA-Binding Specificities of the *Drosophila* hnRNP A/B Family Members

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## SUMMARY

Heterogeneous nuclear ribonucleoproteins (hnRNPs) have been traditionally seen as proteins packaging RNA nonspecifically into ribonucleoprotein particles (RNPs), but evidence suggests specific cellular functions on discrete target pre-mRNAs. Here we report genome-wide analysis of alternative splicing patterns regulated by four *Drosophila* homologs of the mammalian hnRNP A/B family (hrp36, hrp38, hrp40, and hrp48). Analysis of the global RNA-binding distributions of each protein revealed both small and extensively bound regions on target transcripts. A significant subset of RNAs were bound and regulated by more than one hnRNP protein, revealing a combinatorial network of interactions. In vitro RNA-binding site selection experiments (SELEX) identified distinct binding motif specificities for each protein, which were overrepresented in their respective regulated and bound transcripts. These results indicate that individual heterogeneous ribonucleoproteins have specific affinities for overlapping, but distinct, populations of target pre-mRNAs controlling their patterns of RNA processing.

## INTRODUCTION

The majority of the genes in higher eukaryotic genomes are interrupted by noncoding sequences, introns, that are removed to allow the coding sequences, or exons, to be spliced together, producing functional mRNAs. The splicing reaction is performed by a large multicomponent machinery, the spliceosome, consisting of ~200 proteins and five different small nuclear RNAs (snRNAs). One surprising observation that has arisen from the recent sequencing of higher eukaryotic genomes is that there is little correlation between the number of genes in a given

genome and organismal complexity. It has been proposed that the paucity of genes in higher eukaryotes could be compensated by the production of different mRNA isoforms, in which portions of the initial pre-mRNA are differentially included in the final mRNA through a mechanism called alternative splicing of pre-mRNA. Alternative splicing has been found to be almost ubiquitous among multiexon genes in mammals (Wang et al., 2008). These mRNA isoforms frequently encode different proteins and can differ in their 5'- or 3'-untranslated regions (UTRs) to include *cis*-acting elements required for the correct expression of the corresponding protein (for review, see Hughes, 2006).

Most of our understanding of the mechanisms controlling pre-mRNA splicing comes from molecular approaches focused on single-gene studies. These studies have led to the molecular characterization of only a relatively small number of alternatively spliced genes but provide a general understanding of the basic mechanisms of splicing and its regulation.

The sequences recognized by the splicing machinery are highly degenerate and frequently embed in introns that are significantly larger than the flanking exons, and they necessitate auxiliary proteins to promote their use. Conversely, several protein factors have been found to modify the interaction of the splicing machinery with the pre-mRNA. Several of these regulatory protein factors have been characterized and found to recognize *cis*-acting elements within pre-mRNAs that generally fall into two major categories, splicing enhancers and splicing silencers, found in either exons or introns (reviewed in Black, 2003; Cartegni et al., 2002; House and Lynch, 2008; Pagani and Baralle, 2004; Smith and Valcarcel, 2000).

Two groups of proteins with apparently antagonistic effects on splicing regulation have been well characterized to date: the SR family and the hnRNP proteins. Several SR proteins recognize splicing enhancers and stimulate the use of nearby splice sites through interactions with components of the basal splicing machinery, like the U2AF factor and the U1 snRNP 70K protein (for review, see Matlin et al., 2005). hnRNP proteins, which have more than 20 different members in most mammals and 10–15 members in *Drosophila* (Dreyfuss et al., 2002), tend to

interact with splicing silencers (for review, see [Matlin et al., 2005](#)). Some hnRNP proteins prevent binding of other splicing factors to the pre-mRNA by forming complexes with RNA and/or other proteins and prevent the association with RNA splicing control elements ([Zhu et al., 2001](#)). hnRNP proteins also mediate long-range interactions between distant RNA regions flanking alternative exons, thus looping out the intervening region of the pre-mRNA and preventing splicing of the excluded RNA region ([Blanchette and Chabot, 1999](#); [Martinez-Contreras et al., 2006](#)). In several cases, hnRNP proteins have been found to antagonize, both in vitro and in vivo, the activity of SR proteins ([Mayeda et al., 1993](#); [Zahler et al., 2004](#); [Zhu et al., 2001](#)).

High-density oligonucleotide microarray technologies provide the opportunity to profile RNA splicing patterns and the interaction of RNA-binding proteins with RNA transcripts at the whole-transcriptome level, which offers an insight into the post-transcriptional control of gene expression at the level of mRNA maturation and alternative splicing (for review, see [Blencowe, 2006](#)). We have developed a splicing-sensitive microarray platform to monitor changes in alternative splicing in *Drosophila* ([Blanchette et al., 2005](#)). Our first analysis identified the alternatively spliced genes controlled by four different splicing regulators—two SR proteins, SC35 and B52/SRp55, and two hnRNP members from different protein families, PSI and hrp48. This initial analysis suggested that very few genes are coregulated by these SR proteins and hnRNP proteins, while identifying a significant number of genes coregulated by members of the same protein family. These experiments raised several important questions, namely the extent of coregulated genes within a family of splicing regulators; the specificity, organization, and distribution of the *cis*-acting elements recognized by those proteins; and the molecular mechanisms used to regulate these alternative splicing events.

The hnRNP A/B family of proteins is well conserved from *C. elegans* to humans and is believed to carry out similar functions across animal phyla. In this work, we have studied four closely related members of the five known *Drosophila* hnRNP A/B family: hrp36, hrp38, hrp40, and hrp48. Using splicing-sensitive microarrays, between 200 and 300 genes were detected as specifically regulated by each individual hrp protein. The specific RNA sequence motifs recognized by individual hrp proteins were identified using purified proteins and in vitro selection (SELEX) and found to be different for all four proteins tested. The identified binding sites were significantly overrepresented in the genes specifically, as well as coregulated by the four hnRNP proteins, indicative of a complex regulatory network. The genome-wide RNA-binding distributions of the four hrp proteins were characterized using a newly developed nuclear RNA-protein (RNP) complex immunopurification approach in conjunction with *Drosophila* whole-genome tiling arrays (RIP-Chip). Discrete binding locations on expressed RNA transcripts were characterized for all four proteins and found to be predominantly intronic for hrp36, hrp38, and hrp40, while largely exonic binding regions were found for hrp48. Finally, similarly to what was found in our previous analysis, there was no significant overlap between genes regulated by the four hrp proteins and two of the eight different *Drosophila* SR proteins. In summary, our data suggest that hnRNP proteins bind and control alternative

splicing of specific subsets of pre-mRNAs through sequence-specific binding to their target RNAs. Together, these studies provide mechanistic insights into how these subsets of specific pre-mRNAs are regulated in vivo.

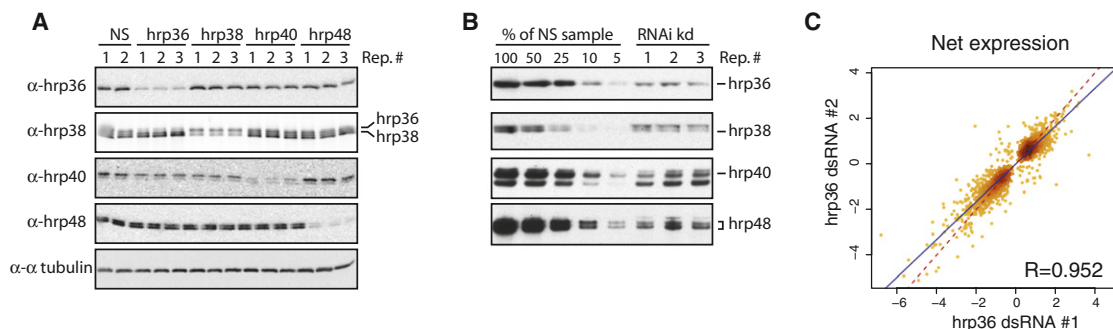
## RESULTS

### HnRNP Proteins Regulate Both Unique and Overlapping Subsets of Splice Junctions

Several hnRNP proteins have been found to specifically regulate the alternative splicing patterns of transcripts in all metazoans. hnRNP A1 is one of the best-characterized hnRNP proteins and is part of a larger family of related proteins, the hnRNP A/B RNA-binding proteins ([Dreyfuss et al., 2002](#)). Previous reports suggest overlapping activities among the mammalian family members ([Bilodeau et al., 2001](#); [Caputi et al., 1999](#); [Hutchison et al., 2002](#); [Mayeda et al., 1994](#)) as well as the *Drosophila* hnRNP A/B family members ([Zu et al., 1996](#)). In order to examine the extent of this overlap, we used a microarray-based approach ([Blanchette et al., 2005](#)) to globally identify the specific splicing events on target transcripts regulated by four highly related members of the fly hnRNP A/B family, namely hrp36, hrp38, hrp40, and hrp48 (see [Figure S1](#), available online, for a description of the proteins and their degree of similarity to each other).

To accommodate the release of Flybase version 4.2.1, a redesigned microarray was used to interrogate the 2,797 annotated *Drosophila* genes having EST evidence of alternative splicing (see the [Experimental Procedures](#) and the [Supplemental Data](#)). These alternatively spliced genes are predicted to produce on average 2.82 isoforms per gene to generate 7,892 different mRNAs corresponding to 9,434 and 10,676 alternative and constitutive exon-exon junctions simultaneously monitored. First, we investigated the effect of changing the levels of specific hnRNPs on global splicing patterns in *Drosophila* cell culture. Expression of specific splicing regulators was reduced by RNAi knockdowns in three independent replicates, and the splice junction abundance was profiled using the splicing-sensitive microarrays. The net expression of all mRNA splice junctions was calculated, and, after population normalization and averaging of the biological replicates, the junctions whose abundance was significantly affected in the knockdown samples (two or more standard deviations from the population net expression average) were grouped together. Reduced expression of hrp36, hrp38, hrp40, and hrp48 was confirmed by immunoblot analysis for each protein ([Figure 1A](#)). The level of knockdown, evaluated to be around or greater than 90% of the endogenous level, was similar between the triplicate samples and between the different hnRNPs tested ([Figure 1B](#)). In addition, the specificity of the knockdowns can be seen by the absence of appreciable changes in expression of the other three hnRNP proteins in each knockdown ([Figure 1A](#)). Finally, using hrp36 knockdown as a test case, no major off-target effects were detected, as the overall changes in net expression between two different nonoverlapping dsRNAs were very similar ([Figure 1C](#), Pearson correlation coefficient, 0.952).

Using the splicing-sensitive microarrays, we found that the numbers of significantly affected splice junctions in the knockdowns of hrp36, hrp38, hrp40, and hrp48 were estimated to be



**Figure 1. Knockdown of Expression of Four Members of the *Drosophila* hnRNP A/B Family by RNAi in *Drosophila* S2 Cells**

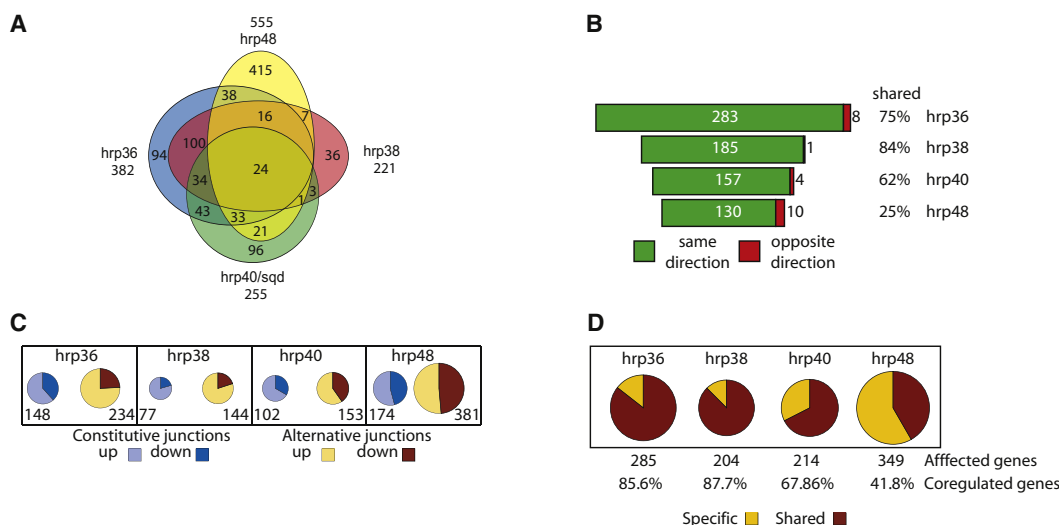
(A) Western blot analysis shows that hrp36, hrp38, hrp40, and hrp48 are efficiently knocked down in triplicate samples as compared to cells treated with a nonspecific dsRNA (NS).  $\alpha$ -tubulin is used as a control to confirm that similar levels of proteins were loaded.

(B) Titration of the protein sample treated with nonspecific dsRNA is used to evaluate the knockdown efficiency.

(C) The overall changes in the net expression value is similar for cell knockdown in the hrp36 expression with two different nonoverlapping dsRNAs. A splicing junction in one knockdown is plotted against the same splicing junction in the second knockdown. The relative feature density is expressed as a darker shade of yellow. The blue line is the fitted linear model used to calculate the R value. The dashed red line corresponds to a perfect correlation.

382, 221, 255, and 555, respectively (Figure 2A), with a validation rate estimated by RT-PCR to be around 70% (Figure S2). These numbers are similar to what we had previously found for RNAi knockdowns of the *Drosophila* SR proteins, dASF/SF2 and B52/SRp55 (Blanchette et al., 2005). We found that both alternatively and, to a lesser extent, constitutively spliced junctions were affected (Figure 2C), suggesting that either the current annotation does not account for all the different alternatively spliced mRNAs or that several cryptic splice sites are being used when the expression level of a given hnRNP protein is reduced.

We next asked whether a significant number of regulated splicing events were under the control of more than one individual hrp protein. We found that hrp36 and hrp38 shared the vast majority of their regulated junctions with the other hrp proteins (Figures 2A and 2B; 75% and 84% of the affected junctions, respectively), while hrp48 shared the lowest number of junctions (Figures 2A and 2B; 140 junctions or 25% of the affected junctions). Interestingly, the vast majority of the junctions coregulated by more than one hnRNP proteins (more than 93% in every case) were affected in the same direction



**Figure 2. Genome-wide Identification of Splice Junctions Regulated by Individual Members of the *Drosophila* hnRNP A/B Family**

(A) Venn diagram of the extent of overlap of the splice junctions regulated by each individual hnRNP protein.

(B) Representation of the coregulated junctions for each splicing regulator demonstrating that most of the coregulated junctions are affected in the same direction (green) or the opposite direction (red) together with the fraction of the junctions coregulated for each individual hnRNP protein.

(C) Breakdown of the number of junctions found to be significantly affected in each individual RNAi knockdown. Constitutive junctions are shown in light blue (up) and dark blue (down). Alternative junctions are shown in light orange (up) or red (down).

(D) Number of genes with junctions regulated by a single hnRNP protein (specific, light yellow) together with the fraction of genes coregulated by a second hnRNP protein (shared, red).

(i.e., a junction going up in a given knockdown also goes up in the other knockdowns and vice versa; Figure 2B).

Using the current splicing-sensitive microarray, using a cutoff of two standard deviations, the number of alternatively spliced transcripts regulated by hrp36, hrp38, hrp40, and hrp48 in Schneider Line 2 cells (S2) is estimated to be 285, 204, 214, and 349 genes, respectively (Figure 2D). Using FatiGO (Al-Shahrour et al., 2004) to assess over- or underrepresentation of gene ontology terms in the populations of genes regulated by the different hnRNP proteins, no significant functional clustering was detected in the genes regulated by hrp36, hrp38, and hrp40, while only a small number of genes involved in neuronal development showed a significant overrepresentation in the hrp48-affected genes (data not shown and Table S1). This suggests that the *Drosophila* hnRNP A/B homologs regulate broad and unrelated sets of genes.

### Different hnRNP Members Associate with Different RNA Subsets

Analysis of exon-junction utilization in different knockdown environments suggests that different members of the *Drosophila* hnRNP A/B family regulate alternative splicing of distinct, but overlapping, sets of target pre-mRNAs. One question raised by this observation is whether different proteins that share a target RNA bind to the same region or to different locations. We used antibodies specific to the four hnRNP proteins to immunopurify RNAs from nucleoplasmic ribonucleoprotein (RNP) particles containing each protein (Figure S3) and determined their genomic locations on the pre-mRNAs isolated from each purification using a 35 nt tiling array. The RIP-Chip approach (RNA IP detected on DNA Chip) was first validated by confirming the presence of six previously known targets of hrp48 in the immunopurified RNA as well as tracts of significantly overrepresented signals on the tiling array data (Figure S4). In addition, 20 randomly selected RNA regions bound by hrp36 on the tiling array were selected, and their level of enrichment in immunopurified RNA was compared to the starting RNP extract. The RNA abundance relative to three different highly expressed but unbound genes, Rps15, Hsc70-4, and Ef2b, was evaluated by real-time RT-PCR from two individually hrp36-immunopurified RNA samples and compared to two RNA samples isolated from the starting RNP extract. The real-time RT-PCR analysis shows a significant enrichment of 18 out of the 20 regions predicted to be bound by hrp36 in all three comparisons (Figure S5, validation rate of 90%).

We found that hrp36, hrp38, hrp40, and hrp48 bind to RNAs that map to 5658, 2286, 4001, and 1359 regions of the *Drosophila* genome, respectively (Table 1). Within these bound regions, hrp36 binds to the largest number of genes (2410 different genes; Table 1), while hrp48 binds to only 341 different genes (Table 1). The number of genes bound by hrp38 and hrp40 was intermediate between these two extremes—1219 and 1922 different genes, respectively. Interestingly, intronic sequences were mainly found associated with hrp36, hrp38, and hrp40 (54.1% for hrp36 and hrp38 and 50.9% for hrp40), while, in contrast, hrp48 was found to bind preferentially to exonic sequences (44.9%; Table 1). Intergenic sequences, although to a lesser extent, were also found associated with all four hnRNP

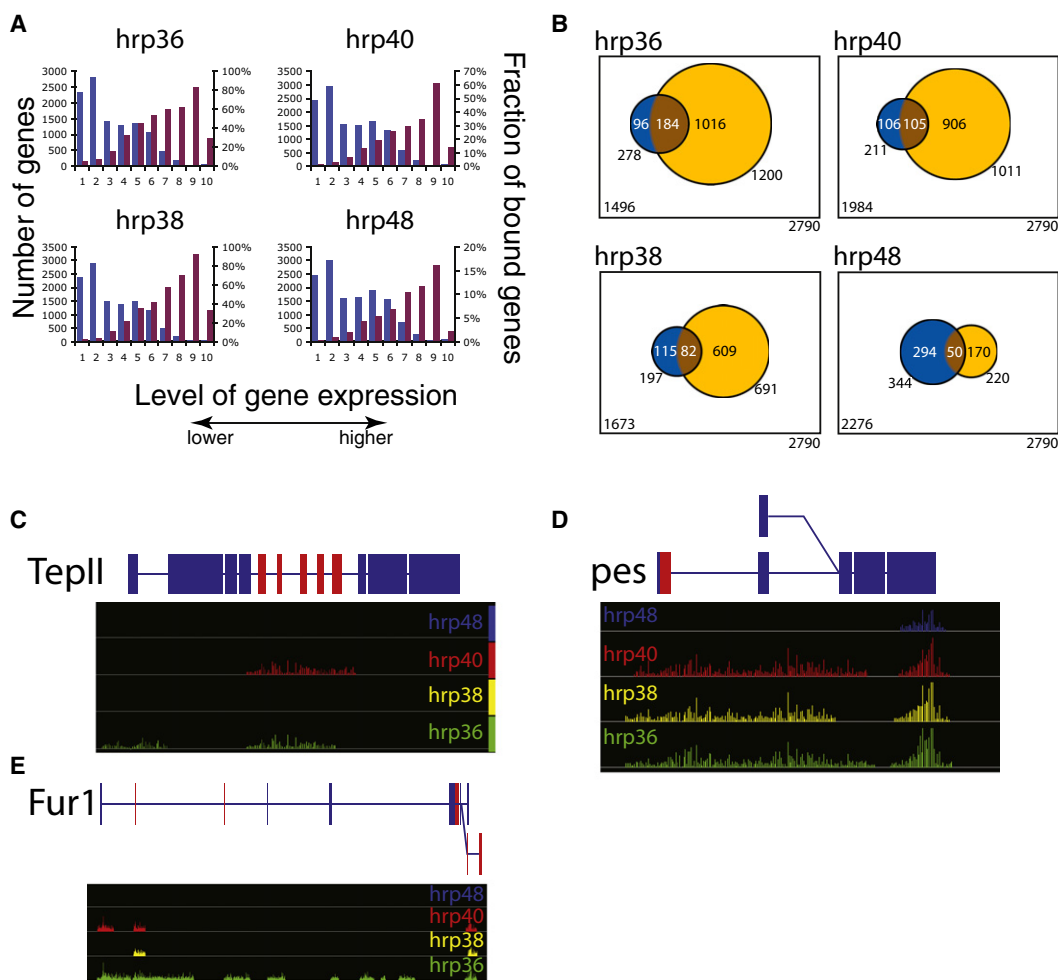
**Table 1. Statistics of the hrp Protein Binding Tracts Identified on the Tiling Arrays by RIP-Chip Analysis**

	Number of Binding Regions Found	Fraction of Regions Found in			Number of Genes Bound
		Exons	Introns	Intergenic	
hrp36	5658	20.8%	54.1%	22.1%	2410
hrp38	2286	23.1%	54.1%	22.8%	1219
hrp40	4001	24.7%	50.9%	24.3%	1922
hrp48	1359	44.9%	22.1%	33.0%	341

proteins. Between 7.3% for hrp48 and 11.7% for hrp36 of the tract (Table 1) comes from intergenic sequences disconnected from nearby annotated genes and might represent unannotated genes or, most likely, unannotated remote 5' or 3' exons connected to distant genes (Manak et al., 2006). These observations, together with the fact that hrp48 shares the least number of regulated splice sites, raise the possibility that hrp48 has more distinct and specialized functions than the other three *Drosophila* hnRNP A/B family members.

Further, we determined the abundance of all expressed mRNAs in *Drosophila* S2 cells using Affymetrix GeneChip *Drosophila* Genome 2.0 arrays and the fraction of genes bound by a given hnRNP protein. If, as is often assumed, hnRNP proteins bind RNA nonspecifically (Dreyfuss et al., 2002 and references therein), there should be a strong correlation between the representation of a specific mRNA in the immunopurified material and the abundance of that mRNA in the starting RNP fraction. Contrary to this notion, we found that the most highly expressed genes (Figure 3A, higher 10 percentiles) are not as frequently bound as their less-expressed counterparts (Figure 3A, percentiles 30–90). With the exception of genes that are not expressed or expressed at very low levels (Figure 3A, lower 30 percentiles), the fraction of genes bound by each individual hnRNP is very similar (Figure 3A). This observation strongly suggests that the four members of the *Drosophila* hnRNP A/B family of proteins bind to distinct, but only partially overlapping, populations of mRNAs in a selective manner.

Examination of individual genes provides diverse examples of differential binding by hnRNP proteins. For example, the alternatively spliced Fur1 pre-mRNA shows discrete binding for hrp36, hrp38, and hrp40, where some of the bound regions overlap, while other transcript regions are specific to individual proteins (Figure 3E). A very different outcome is observed on the pre-mRNA encoding the alternatively spliced TepII and pes genes, where binding of the different hnRNP proteins is contiguous over multiple regions (Figures 3C and 3D). These and other examples support the specific nature of interaction between hnRNP A/B family members and individual pre-mRNAs in *Drosophila*. In addition, the strong bias of hrp36, hrp38, and hrp40 for intronic RNA is additional evidence of the specific nature of their RNA-binding properties; introns are short-lived, nuclear RNA species, and they are not expected to be preferentially associated and recovered with proteins with low RNA-binding specificity.



**Figure 3. Genome-wide Identification of the RNAs Associated with the Different hnRNP Proteins in RNP Complexes**

(A) Distribution of the level of gene expression measured by Affymetrix arrays measured in S2 cells (blue bar) and the fraction of RNA bound by a hnRNP protein within a given level of expression (purple bar).

(B) The overlap between the genes that were found regulated in the splice junction microarray (blue) and bound in the tiling array (yellow). The highly significant overlapping transcripts are represented in brown (Fisher's exact test,  $p$  value  $< 2 \times 10^{-5}$ ).

(C and D) TepII and pes, two representative genes bound by hnRNP proteins, showing extensive coverage suggestive of the spreading model.

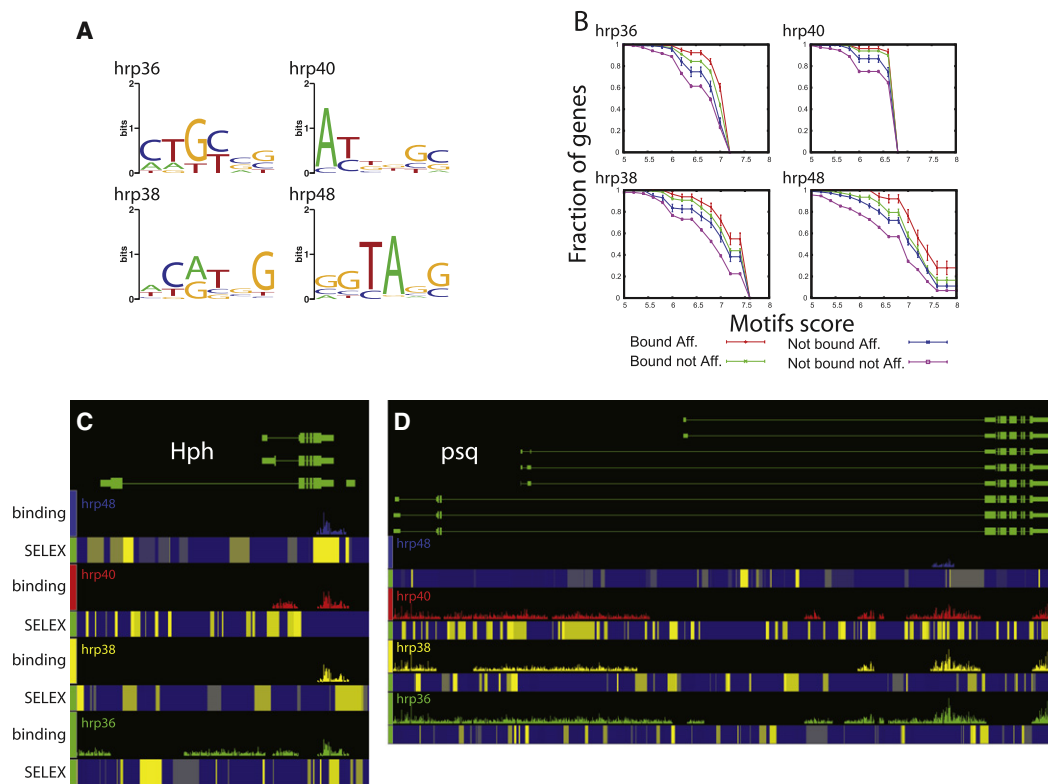
(E) Fur1, a representative gene bound by hnRNP proteins, showing discrete intronic binding tracts for hrp40 suggestive of the looping model. Significantly over-represented signals in the IP samples over the signal measured from RNA extracted from the starting RNP extract are represented by bars along the genome, with the eight of the bar corresponding to  $p$  values (expressed as  $-\log [p]$ ). Above is a representation of the gene structure; blue boxes are constitutive exons, red boxes are alternative exons, and lines are introns. Pes has two alternative promoters, while Fur1 has two alternative poly(A) signals represented by the two levels in the gene structure.

We next sought to determine whether binding of a given hnRNP protein to an RNA transcript correlates with the ability of that hnRNP protein to regulate alternative splicing of the bound pre-mRNA. We found an extensive overlap between genes with RNAs found associated with a given hnRNP protein on the tiling arrays and alternative pre-mRNA splicing regulated by the same protein on the splicing-sensitive microarrays (Fisher's exact test  $p$  value  $< 2 \times 10^{-5}$ ; Figure 3B). These results support the notion that the specific binding of a given hnRNP protein to a pre-mRNA is likely to be associated with the control of alternative splicing of the bound pre-mRNA.

#### Binding Site Specificity of the Different Members of the *Drosophila* hnRNP A/B Protein Family

Our experiments show that 270 genes were regulated by more than one hnRNP protein, and also that 1888 RNAs were bound by more than one hnRNP protein. What remained unknown was whether different hnRNP proteins acting on the same target pre-mRNA interact with the same or different *cis*-acting RNA elements. To address the RNA-binding specificities of the hrp proteins, we performed in vitro selection of binding sites (SELEX) on recombinant hrp36, hrp38, hrp40, and hrp48 proteins. Following eight rounds of selection, individual bound RNAs were cloned and sequenced. Sequences were aligned, and the





**Figure 4. HnRNP Proteins Bind to Different and Specific RNA Motifs that Are Overrepresented in the Genes Regulated and Bound by the Respective hnRNP Proteins**

(A) Graphical representation of the preferred binding motif identified by SELEX for each individual hnRNP protein (Crooks et al., 2004). Height of each bar shows the information content at each position of the binding motif in bits (log-odds in base 2).

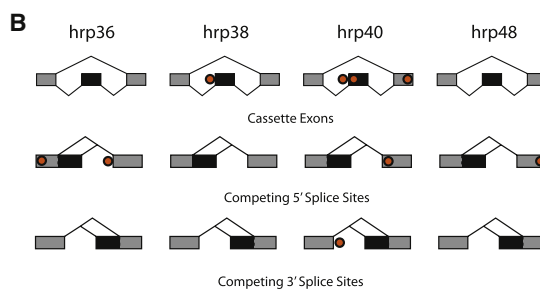
(B) Cumulative distribution of the presence of at least three occurrences of the SELEX motif in the genes identified as being regulated by the splice junction array or identified as bound by the tiling array. Error bars are determined analytically using the binomial distribution and correspond to standard deviation of  $1: \sqrt{np[1-p]/n}$ , where  $n$  is the number of sequences searched and  $p$  is the observed probability of having sites of given score.

(C and D) Hph and psq, two representative genes showing that the binding tracts identified on the tiling arrays (colored bar tracts) are frequently associated with clusters of high-affinity binding sites. A moving window using the SELEX motifs was used to calculate the score at each position along the fly genome. The motif score at a given position is represented as a heat map—blue, low motif score; yellow, high motif score.

common motifs in them were identified using MEME (Bailey and Elkan, 1994; Figure 4A). The models of the most overrepresented sites derived for each of the RNA-binding proteins appear to be unrelated to each other (Figure 4A). Comparison of these models to the *Drosophila* transcriptome revealed a significant overrepresentation of each of these preferred binding sites in the population of genes regulated by the corresponding hnRNP protein as compared to the rest of the genes queried by the splicing-sensitive microarray analyses (Figure S6). If we examined the subset of genes that were both found in a complex with a given hnRNP protein and exhibited differential splicing in the cell lines where the same protein was knocked down, the extent of enrichment in the corresponding preferred binding site was even higher (Figure 4B, red lines). Moreover, in all cases tested, the second- and third-largest fractions of genes having high scores for the preferred SELEX binding motifs were the genes found to be bound only by the corresponding hnRNP protein and found to only exhibit differential splicing in the RNAi knockdown experiment, respectively (Figure 4B, green and blue lines, respectively). Finally, in all four cases studied here, the populations of genes

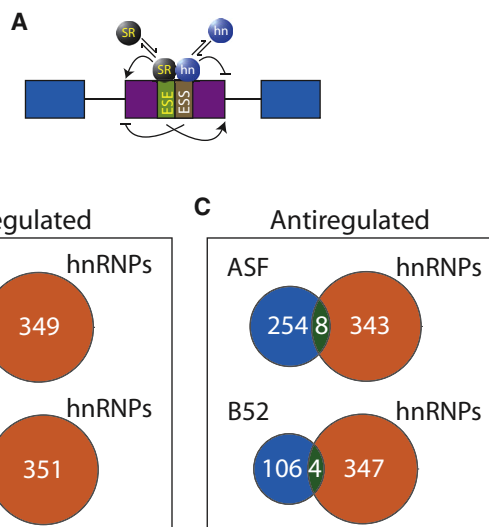
that were neither bound nor regulated by a specific hnRNP proteins had the lowest fraction of genes with high-scoring in vitro-selected binding sites for the corresponding hnRNP protein (Figure 4B, purple lines). This series of observations provides further evidence that each hnRNP protein regulates alternative splicing through the recognition of specific and different binding sites within its target pre-mRNAs and that multiple hnRNP proteins may simultaneously target the same pre-mRNA through distinct binding sites.

The preferred RNA-binding motifs identified in the SELEX experiments can be used to search for putative binding sites within the RNA regions identified in the RIP-Chip analysis. In many cases, exemplified by the Hph and psq genes, one or more of the respective preferred binding motifs are indeed found in the regions covered by hnRNP proteins (Figures 4C and 4D). The gene regions identified on the tiling arrays following immunoprecipitation of the hnRNP complexes are often found to contain clusters of the preferred SELEX motifs (Figures 4C and 4D and Figure 5A for a nucleotide level blowup and Figure S7). Among the genes bound by multiple hnRNP proteins, we noted that



the high-scoring motifs for a given hnRNP protein are not always evenly distributed along the bound region. Frequently, the bound regions are found associated with clusters of the preferred binding motifs of coassociated hnRNP proteins, as with Hph and psq (Figures 4C and 4D and Figure 5A). These observations strongly suggest the involvement of cooperative binding among different hnRNPs to form the mature RNP particles (Domsic et al., 2003; Zhu et al., 2001). This cooperative arrangement of low-affinity and high-affinity binding sites for different proteins is reminiscent of the architecture of transcription factor-binding sites in eukaryotic transcriptional enhancers (Papatsenko et al., 2006; Zeitlinger et al., 2007; Zinzen et al., 2006).

Knowing the target transcripts and specific splice junctions affected by RNAi to each of the four hnRNP proteins and the RNA-binding site motifs from the SELEX experiments, we asked if there was enrichment of the SELEX motifs near the affected splice junctions. We have previously performed a similar analysis for the two SR proteins, B52/SRp55 and dASF/SF2 (Blanchette et al., 2005), and similar studies were done with mouse Nova-1/2 target transcripts (Ule et al., 2006). Here, the affected splice events were grouped according to splicing patterns (cassette exon, competing 5' splice sites and competing 3' splice sites). This analysis looked for the enrichment of preferred binding site scoring matrices within windows adjacent to affected splice events compared to splice events not affected by the hnRNP (see the Experimental Procedures and the Supplemental Data). Positions enriched with hnRNP-binding sites are shown in Figure 5B (also Figure S8). These findings suggest a preferential binding site location for a given factor controlling a specific splice event, as seen previously (Aznarez et al., 2008; Ben-Dov et al., 2008; Blanchette et al., 2005; Castle et al., 2008; Licatalosi et al., 2008; Ule et al., 2006; Zhang et al., 2008). However, none of the enrichments were significant after using a conservative Bonferroni correction for >2000 comparisons between affected splicing events and controls. Thus, while the target transcripts affected by RNAi against a given hrp protein appear to be enriched in the SELEX motifs for that factor (see Figure 5A for an example), there does not seem to be a strict fixed location at which a given factor acts on a specific class of splicing events (Figure 5B). Other studies have shown a complex relationship between the location of splicing control elements and their activities (Goren et al., 2006; Wang et al., 2006), and this lack of a strict spacing of regulatory elements may reflect differences in the mechanisms of action of the *Drosophila* hnRNP A/B family of proteins (Cartegni et al., 2002) and the KH domain splicing factor, Nova-1 (Ule et al., 2006). This variability in the spacing of regulatory elements is also common in transcriptional enhancers and silencers (Levine and Tjian, 2003). Our bioinformatic analysis also does not take into account the possible role of weak or non-consensus binding sites, which could be important in the context of cooperative interactions with other splicing factors and might



**Figure 6. No Significant Antagonistic Regulation Is Found between Two SR Proteins and Four hnRNP A/B Family Proteins in a Single Defined Cellular Environment**

(A) It has been suggested that several genes are under the antagonistic control of SR and hnRNP proteins, in which the pattern of splice isoforms is dependent on the relative concentrations of SR and hnRNP proteins.

(B) Comparison of the genes that are found to be regulated in the same direction between hnRNPs (this analysis) and two different SR proteins (Blanchette et al., 2005).

(C) Comparison of the genes that are found to be regulated in opposite directions between hnRNPs (this analysis) and two different SR proteins (Blanchette et al., 2005).

explain the lack of significant enrichment of strong binding sites within fixed windows near affected splice junctions.

### Control of Alternative Splicing by hnRNP and SR Proteins

A model of antagonistic control of alternative splicing, mediated by the relative levels of hnRNP and SR proteins, has been proposed (Cáceres et al., 1994; Eperon et al., 2000; Mayeda et al., 1993; Mayeda and Krainer, 1992; Zhu et al., 2001; Figure 6A). We compared the results of our current analysis with the data generated from a previous study in which the expression of two SR proteins, dASF/SF2 and dSRp55/B52, was knocked down (Blanchette et al., 2005). The most important conclusion from our previous comparison was that the SR and the two different hnRNP protein knockdowns affected distinct sets of splice junctions. With a very few exceptions, a similar conclusion was reached when this previous analysis was extended to include the four *Drosophila* hnRNP A/B homologs. Namely, very few splice junctions were affected in the same and opposite directions (Figures 6B and 6C), suggesting that,

**Figure 5. Locations of Binding-Site Enrichment for Each hnRNP Associated with Specific Alternative Splicing Patterns**

(A) Magnification of Hph binding tracts showing a 20,000 nt long region (middle panel) to the nucleotide level showing the underlying genomic sequence (bottom of the window) with the presence of the SELEX motifs (colored bar above the sequence) corresponding to the hrp48- and hrp40-recognized motifs (cyan and magenta boxes, respectively).

(B) Circles show positions that had an enrichment of SELEX motifs in affected splicing events (two-sided Wilcoxon ranked-sum test,  $p < 0.01$  without multiple trials correction for >2000 trials) using any of several measures for enrichment (see the Experimental Procedures and Figure S8 for details).



at least in the *Drosophila* cell culture system with the two SR proteins examined here, an antagonistic relationship of SR and hnRNP proteins in alternative splicing is extremely rare. This issue needs to be addressed further in vivo in other model organisms, especially in mammals.

## DISCUSSION

In this study, we report a genome-wide analysis of control of alternative splicing by four *Drosophila* homologs of the mammalian hnRNP A/B proteins, a family of proteins that has been implicated in a variety of cellular processes including telomere biogenesis, mRNA localization, translation, and stability (reviewed in Krecic and Swanson, 1999). By interrogating each predicted splice site by specific molecular probe in an array format, we have found that the four different *Drosophila* hnRNP A/B protein homologs regulate alternative splicing of specific, but partially overlapping, sets of pre-mRNAs. We also found that a majority of the targets of the hrp36, hrp38, and hrp40 proteins were coregulated by more than one member of this protein family, while hrp48 regulated a larger and more distinct subset of pre-mRNAs. Moreover, RIP-Chip experiments indicate that the former three proteins bind predominantly intronic regions of pre-mRNA, while hrp48 predominantly binds to exonic sequences, in both discrete and more extensive transcript regions. The preferred RNA-binding motifs for each individual protein, determined by the SELEX method, were different for each protein, and the transcripts regulated and bound by each factor were found to be enriched in the corresponding binding sites.

### hnRNP Proteins and Splicing Silencer Function: Spreading or Looping?

Different models have been proposed to explain the molecular mechanisms by which hnRNP proteins control alternative splicing at splicing silencer elements. Our RIP-Chip data show the binding distributions of the hrp proteins on target transcripts and can be used to address the models for how splicing silencers are controlled by hnRNP proteins. It had first been proposed that hnRNP A1 controls its own alternative pre-mRNA splicing by a mechanism involving protein-protein interactions between distantly bound A1 protein at high-affinity binding sites. These distant binding sites would bring the widely separated splice sites in close proximity while preventing utilization of the more proximal splice sites, presumably by looping out of the alternative exon leading to exon skipping (Blanchette and Chabot, 1999). This model, involving protein-mediated looping, also predicted that hnRNP A1-binding sites should be able to act as splicing enhancers to promote intron definition when the intron is large, which was recently documented (Martinez-Contreras et al., 2006; Nasim et al., 2002). Consistent with the idea of hnRNP-mediated looping, several discrete binding regions that are distantly spaced and flank alternative exons can be found on specific pre-mRNAs, like on the Fur1 pre-mRNA (Figure 3E), suggesting that the *Drosophila* hnRNP A/B members could regulate alternative splicing in vivo by a mechanism similar to the looping model defined by biochemical experiments in vitro. More recently, a different model has also been proposed

to explain how mammalian hnRNP A1 regulates alternative splicing. It has been proposed that hnRNP A1 binding to exonic regions prevents binding of SR proteins to their cognate splicing enhancers by nucleating and spreading over extended regions of the pre-mRNA via cooperative RNA binding to weak adjacent sites, which is also mediated by protein-protein interactions between hnRNP proteins (Domsic et al., 2003; Zhu et al., 2001). Consistent with the spreading mechanism, large regions of pre-mRNA were found to be associated with the four different *Drosophila* hnRNP proteins, as in the TepII and pes pre-mRNA (Figures 3C and 3D), suggesting that a similar nucleation mechanism might also be used by this family of splicing factors to control alternative splicing events. Interestingly, we also see that local smaller regions bound by one hnRNP protein can be found within a larger region covered by a different hnRNP protein, as seen for Furl, in which discrete regions of hrp40 binding are found within a large portion of this pre-mRNA that is bound by hrp36. These observations suggest that both biochemical models might actually function together and provide in vivo evidence supporting both the spreading and the looping models for splicing silencer function.

### An RNA Splicing Code

Recently, several genome-wide surveys of the patterns of alternative splicing across different mammalian tissues have identified known or novel RNA sequence motifs associated with specific differential splicing events (Castle et al., 2008; Ule et al., 2006; Wang et al., 2008). It is now becoming clear that specific splicing regulators rely on an RNA code in their target transcripts and that this RNA code is responsible for establishing the cellular constellation of differentially spliced mRNAs (Aznarez et al., 2008; Castle et al., 2008; Licatalosi et al., 2008; Wang et al., 2008; Zhang et al., 2008). However, very few and only individual factors regulating alternative splicing have been globally linked to their *cis*-acting regulatory elements (Aznarez et al., 2008; Licatalosi et al., 2008; Zhang et al., 2008). In this analysis, we found that a large proportion of the pre-mRNAs regulated by either hrp36, hrp38, hrp40, or hrp48 were also regulated by at least one additional hnRNP protein. However, even though their protein sequences are similar (Figure S1), our studies showed that the RNA motifs recognized by each hrp protein are distinct. Nonetheless, their binding locations observed across target transcripts are extensively overlapping, which raises the possibility that some hnRNP A/B family proteins may have redundant functions at individual splice junctions. Interestingly, in *Drosophila*, single hrp36 or hrp38 mutants are viable, but combining mutations in both genes causes synthetic lethality (Haynes et al., 1991; Zu et al., 1996; S. Haynes, personal communication), which is consistent with the largely overlapping molecular data sets of bound RNAs and regulated splice junctions identified here. By contrast, individual hrp40, also known as squid (sqd), and hrp48 mutations are lethal (Hammond et al., 1997; Norvell et al., 1999).

It is also interesting to note that both hrp36 and hrp38 overexpression were able to affect the alternative splicing of *dopa decarboxylase* (*Ddc*) in vivo (Shen et al., 1995; Zu et al., 1996, 1998) and that no change in *Ddc* splicing pattern was observed in the hrp36 null mutant genetic background

(Zu et al., 1996), possibly due to the redundant activity of the hrp38 protein. One alternative explanation for the large fraction of coregulated targets between hrp36, hrp38, and hrp40 is the possibility that they might exert their action through a combinatorial mode of regulation. Several independent pre-mRNAs have been found to be under the control of a complex network of protein-protein and protein-RNA interactions, and it is thought that a given splicing pattern is generated by the combination of these interactions (for reviews, see Matlin et al., 2005; Smith and Valcarcel, 2000). For example, the skipping of a given alternative exon might first require binding of an hnRNP protein that, through protein-protein interactions, may mediate cooperative binding of a second hnRNP protein to lower-affinity binding sites nearby, leading to the repression of splicing of the alternative exon. In this case, the proper control of alternative splicing would require the combination of these two events; the absence of either hnRNP protein would lead to the same phenotype. A combinatorial mode of regulation is consistent with the high frequency of shared binding regions observed in the data from the RIP-Chip analysis, as well as the extensive overlap between the alternative splicing patterns affected by individual hnRNP proteins.

By comparing the pre-mRNAs regulated by the four hrp proteins with data from a genome-wide analysis of the genes regulated by two SR proteins, dASF/SF2 and dB52/SRp55 (Blanchette et al., 2005), we have found that, at least for the SR proteins tested, an antagonistic regulation between SR and hnRNP proteins is not a major mode of regulation in *Drosophila* cell culture. Similarly, overexpression of hrp36 did not affect the overall localization of SR proteins on transcripts expressed in larval polytene nuclei (Zu et al., 1996), suggesting that dramatic changes in the SR/hnRNP ratio do not significantly affect the distribution of SR proteins on pre-mRNAs. However, it is possible that when the level of an hnRNP is reduced—for example, in the case of an RNAi knockdown—sites normally occupied by hnRNP proteins are now freed up and become accessible to binding by SR proteins, resulting in SR-dependent changes in alternative splicing profiles. In fact, an example of this type of situation has been observed in the Dscam pre-mRNA, in which SR protein-dependent ectopic inclusion of multiple exons can only be seen when the level of hrp36 is reduced by RNAi (Olson et al., 2007). Global analysis of pairwise RNAi knockdowns would provide a better understanding of these complex interactions among alternative splicing regulators.

## EXPERIMENTAL PROCEDURES

### Cell Culture and RNAi

Serum-free adapted D.mel2 cells (GIBCO) were maintained at 25°C in *Drosophila* SFM. In vitro transcription, RNA purification, and preparation of double-stranded RNA were done as described (Blanchette et al., 2005). RNAi knockdowns were performed by seeding  $2 \times 10^6$  cells per well in a 6-well plate and adding 10 µg of dsRNA. After 48 hr of incubation, a second round of 10 µg dsRNA was added, and the cells were allowed to grow for another 48 hr before being harvested and the RNA purified using the RNeasy kit following the manufacturer's protocol (QIAGEN).

### Splicing-Sensitive Microarray: Design and Hybridization

Thirty-six nucleotide exon-junction and exon-body probes were selected from all genes from the GadFly 4.2.1 *Drosophila melanogaster* genome annotation

that contained more than one annotated transcript. A complete description of the design and the hybridization procedure and data analysis can be found in the [Supplemental Data](#) and references therein.

### Tiling Array Hybridization

Nuclear RNP fractions from S2 cells were prepared by using a modified version of the mammalian RNP protocol (see the [Supplemental Data](#) for more details; Pinol-Roma et al., 1990). The immunopurification, labeling, and hybridization were done as previously described (Olson et al., 2007). The statistical analysis of the bound regions was performed using the TiMAT analysis suite (<http://bdtncp.lbl.gov/TiMAT/>) essentially as described (Li et al., 2008).

### RNA SELEX

In vitro selection of the RNA-preferred binding motif was performed by using recombinant protein as described elsewhere (Amarasinghe et al., 2001; Fitzwater and Polisky, 1996). A detail description of the procedure can be found in the [Supplemental Data](#).

### Bioinformatic Analysis of Position-Specific Motif Enrichment

Specific alternative splicing events were inferred from the *Drosophila melanogaster* genome annotation and computationally searched for enrichment of binding sites in the hnRNP-affected genes and compared to the rest of the genome for statistically significant overrepresentation. A detail description of the bioinformatic analysis can be found in the [Supplemental Data](#).

### ACCESSION NUMBERS

Microarray data have been deposited with the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) public database under accession number GSE13940 (<http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE13940>).

### SUPPLEMENTAL DATA

The Supplemental Data include supplemental text, one table, and eight figures and can be found with this article online at [http://www.cell.com/molecular-cell/supplemental/S1097-2765\(09\)00066-5](http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00066-5).

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