The Drosophila Heterogeneous Nuclear Ribonucleoprotein M Protein, HRP59, Regulates Alternative Splicing and Controls the Production of Its Own mRNA*

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The Drosophila heterogeneous nuclear ribonucleoprotein M, HRP59, is a nuclear protein that associates co-transcriptionally with pre-mRNA and is necessary for the correct expression of a subset of mRNAs. We show here that the hrp59 pre-mRNA is alternatively spliced to generate two different mRNAs that differ in the presence of exon 3. Exon 3-containing transcripts make up the majority of hrp59 transcripts and encode for the functional protein, HRP59-1. Transcripts that lack exon 3 contain a premature translation termination codon and are targeted to the nonsense mediated decay pathway. We show that exon 3 inclusion is itself inhibited by HRP59 and that changes in the HRP59 protein levels affect the splicing activity of the cell. We propose that the ability of HRP59 to regulate the alternative splicing of its own pre-mRNA serves in a negative feedback loop that controls the levels of the HRP59 protein and maintains the homeostasis of the splicing environment.

Pre-mRNAs associate co-transcriptionally with a variety of RNA-binding proteins known as hnRNPs proteins or mRNP proteins (reviewed in Refs. 1 and 2). hnRNP proteins exist in all eukaryotes and are evolutionarily conserved. More than 20 hnRNP proteins have been identified in human cells, and these have been designated from A1 to U (reviewed in Ref. 2). Although the degree of homology at the amino acid level is not very high, hnRNP proteins from different organisms share the same types of domain and the same domain organization. In Drosophila melanogaster, more than 10 hnRNP proteins have been identified (3–6).

hnRNP proteins are abundant nuclear proteins and they play a general role in the packaging of nascent transcripts into ribonucleoprotein complexes (pre-mRNP). Many hnRNP proteins also have specific functions in gene expression. For instance, in the cell nucleus the mammalian hnRNP K interacts with the transcription machinery and stimulates transcription (7), while in the cytoplasm hnRNP K controls translation initiation of specific mRNAs (8). hnRNP I, also known as polypyrimidine tract-binding protein, plays a role in mRNA stability and mRNA localization (9, 10).

hnRNP proteins are modular and characterized by the presence of one or more RNA-binding motifs and auxiliary domains of different types (reviewed in Ref. 11). Several hnRNP genes generate multiple isoforms through alternative splicing. The significance of these alternatively spliced forms has been elucidated for members of the hnRNP families A/B, D, and I. For example, the stabilities of human hnRNP D isoforms are differentially controlled by the insertion of an alternate exon that regulates their ubiquitin targeting activities (12). Different isoforms of hrp40, a Drosophila hnRNP A/B homolog, play distinct roles in Gurken localization during oogenesis (13). Alternative skipping of exon 11 in the human hnRNP I transcript leads to the production of a mRNA that is degraded by the nonsense-mediated decay (NMD) pathway (14). Exon 11 skipping is promoted by hnRNP I itself, and thus it has been proposed that autoregulation maintains normal levels of hnRNP I expression.

Some D. melanogaster hnRNPs play a role in the regulation of alternative splicing. Overexpression of members of the hnRNP-A/B family in flies induces exon skipping in the dopa-decarboxylase (Ddc) pre-mRNA (15, 16), while the hpr48 protein is involved in P-element pre-mRNA splicing (17, 18).

We have previously studied the hnRNP M proteins of Chronomonas tentans and of D. melanogaster, referred to as HRP59 (19). The HRP59 M proteins in insects and in mammals have a molecular mass of 65–75 kDa and contain three RNA-binding domains of the RRM type. The HRP59 M family in human cells comprises at least six immunologically related isoforms (20, 21). HRP59 in C. tentans is dynamically recruited to transcribed genes and binds co-transcriptionally to pre-mRNA. Immunoelectron microscopy studies in the Balbiani ring genes of C. tentans have shown that HRP59 accompanies the mRNA from the gene to the nuclear envelope and that it is released from the mRNA at the nuclear pore. We have recently identified the preferred RNA targets of HRP59 and identified a purine-rich motif recognized by HRP59 (19). Interestingly, this purine-rich motif resembles an exonic splicing enhancer of the (GAR)n type (reviewed in Ref. 22) and is similar to the binding site for SF2/ASF, an SR protein involved in splicing regulation (23). These observations suggest that HRP59 is involved in pre-mRNA splicing. Further support comes from a study showing that two of the human hnRNP M isoforms,
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referred to as 72/72(M), are associated with pre-mRNA in pre-spliceosomal A complexes (24).

We have explored the possibility that hnRNP M proteins play a role in pre-mRNA splicing by studying the splicing activity of HRP59 in Drosophila S2 cells. We show that the hrp59 pre-mRNA is alternatively spliced and that HRP59 affects the splicing of its own pre-mRNA by promoting the synthesis of a mRNA with a premature stop codon that is a target for NMD. Our results provide a new example of RNA-binding proteins that regulate their own expression by a feedback mechanism in which alternative splicing is coupled to NMD.

**EXPERIMENTAL PROCEDURES**

**RNA Extraction and RT-PCR Analysis**—Total RNA from Drosophila S2 cells was extracted using the RNAqueous kit (Ambion). Reverse transcription was performed with SuperScript-II (Invitrogen) and hexamer primers on 5 or 25 μg total RNA. One-tenth of the reverse transcription product was used for PCR in a 50-μl reaction, using primers described in the supplemental data. The PCR reaction was conducted as follows: 5 min at 95 °C, followed by 30 cycles, each consisting of 45 s at 95 °C, 45 s at 55 °C, and 1.5 min at 72 °C, followed by a final extension phase for 7 min. The up-10 RT-PCR was followed by nesting a 1:50 dilution of the reaction for 25 cycles. A 25-μl portion of each reaction was run on a 1% agarose gel and stained with EtBr or GelStar (Cambrex BioScience). Amplimers were quantified from digital images using Bio-Rad Quantify Analysis software.

**cDNA Cloning and Expression in Drosophila S2 Cells**—cDNAs for overexpression in S2 cells were cloned into the vector pMT-V5-HisB (Invitrogen). Primers used to amplify cDNA (supplemental data) spanned the start and stop codons of the respective genes and had EcoRI, KpnI, or XhoI restriction sites designed into their 5’ and 3’ ends. Amplification of cDNA was carried out using Platinum Taq-Pfx (Invitrogen). S2 cells were stably transfected using the calcium phosphate method, with hygromycin as the selection co-plasmid. cDNA expression was induced with 500 μM cupric sulfate for 48 h and analyzed by Western blotting.

**Cell Fractionation**—6 × 10^6 Drosophila S2 cells were centrifuged, washed with 10 mM Tris, pH 7.4, 140 mM NaCl, and resuspended in 1 ml of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 3 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% glycerol) supplemented with Complete protease inhibitor mixture (Roche Applied Science). The ice-cold cell suspension was then homogenized and centrifuged at 13,000 rpm for 20 min. The supernatant was removed, and the pellet, which contained the nucleoplasmic fraction, was resuspended in 1 ml of lysis buffer and sonicated. 10 μl of the cytoplasmic and the nucleoplasmic fraction were separately boiled in protein sample buffer and analyzed by Western blotting.

**RNA Interference (RNAi) in Drosophila S2 Cells**—Double-stranded RNAs (dsRNAs) against HRP59, dsF2/ASF, UPF1, and GFP were prepared by in vitro transcription from PCR products with T7 promoters on both ends of the ampliers, using the Megascript RNAi kit (Ambion). Two templates were used for hrp59, targeting overlapping regions of the exon 4. The PCR primers are described in the supplemental data. 20 μg of dsRNA was applied to S2 cells every 48 h, and the cells were harvested after 120 h.

**Immunoblot Analysis and Antibodies**—Western blotting to determine HRP59 levels was carried out as described previously (19) using an affinity-purified polyclonal anti-HRP59 antibody. dsF2/ASF levels were measured using a monoclonal anti-SF2/ASF antibody (Zymed Laboratories). A monoclonal anti-V5 antibody (Invitrogen) was used for detection of V5-tagged proteins.

**In Vivo Splicing Assays**—The constitutive expression vector pAc5/V5-HisB (Invitrogen) was used to construct minigenes. Regions flanking the exon 10A/B and exon 11 were amplified from Drosophila S2 genomic DNA using primers as listed supplemental data to create an up-10 minigene. Hrp59 genomic DNA was amplified using three different primer pairs, I, II, III, that spanned overlapping gene regions. Fragment I, which included exons 1 and 2 and introns 1 and 2, was cloned between the KpnI and XbaI sites of the pAc5 vector. The resulting construct was digested with restriction enzymes XbaI and SacII, and Fragment II, which contained exon 3, the first 100 nucleotides of exon 4, and intron 3 sequences, was inserted. The full-length hrp59 minigene was constructed by inserting Fragment III into construct I and II after it had been digested with SacII. All constructs were verified by sequencing.

In vivo splicing assays were carried out using S2 cells and RNAi-depleted S2 cells, which were transiently transfected with 19 μg of minigene DNA. Transient transfection of minigenes was performed according to the Drosophila Expression System Technical Manual (Invitrogen). Total RNA was isolated after 24–48 h and reverse-transcribed. Splicing patterns were analyzed by RT-PCR using vector-specific and gene-specific primers.

**RESULTS**

**The hrp59 pre-mRNA Is Alternatively Spliced**—A recent bioinformatic approach identified two alternatively spliced mRNAs that originate from the hrp59 pre-mRNA in Drosophila (25). We prepared cDNA from Drosophila S2 cells and amplified hrp59 sequences by PCR. We detected a major hrp59 transcript and a less abundant hrp59 transcript of lower molecular mass. We sequenced both PCR products, and the sequence analysis revealed the existence of two alternatively spliced hrp59 mRNAs that differed in the inclusion of exon 3. We sequenced both PCR products, and the sequence analysis revealed the existence of two alternatively spliced hrp59 mRNAs that differed in the inclusion of exon 3. We termed these transcripts hrp59-1 and hrp59-2 (Figs. 1, A and C, and 2C). We concluded that both hrp59-1 and hrp59-2 mRNAs coexist in S2 cells, hrp59-1 being the predominant mRNA. We did not detect the alternative isoform reported by Philipps et al. (25).

Virtual translation of the hrp59-1 and hrp59-2 mRNAs revealed that the skipping of exon 3 introduces a frameshift and results in the presence of a premature termination codon (PTC) at amino acid 214 (Fig. 1B). Interestingly, the stop codon of hrp59-2 precedes the boundary between exons 4 and 5, and the hrp59-2 transcript is therefore a potential substrate for NMD.

**The Majority of hrp59-2 Transcripts Is Degraded by NMD**—To investigate whether the hrp59-2 transcript is targeted to NMD, we depleted UPF1, an essential NMD factor, by RNAi in S2 cells and examined changes in hrp59 mRNA levels by RT-
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PCR (Fig. 1C). We applied the RNAi method described by Clemens et al. (26) based on the use of long dsRNAs. The efficiency of the UPF1 depletion was confirmed by RT-PCR amplification of upf1 mRNA and by the stabilization of an endogenous mRNA, the ornithine decarboxylase antizyme mRNA (oda), known to be regulated by NMD (27). We quantified changes of mRNA levels after UPF1 depletion and determined the fold of enrichment compared with the mRNA levels in control cells treated with GFP dsRNA (Fig. 1C). The UPF1 knockdown caused a significant increase of hrp59-2 mRNA levels relative to the GFP control cells. After five independent experiments, we calculated a 4-fold average enrichment of hrp59-2 mRNA in the UPF1 knockdown. This result indicates that the hrp59-2 mRNA is degraded by the NMD pathway.

The HRP59-2 Isoform Accumulates in the Cytoplasm—Most of the hrp59-2 mRNA is degraded by NMD, but there is a remaining fraction that can be detected by RT-PCR under steady-state conditions in non-treated S2 cells. This remaining hrp59-2 mRNA is probably translated. The predicted protein encoded by the hrp59-2 mRNA contains RRM 1 but lacks RRM 2 and RRM 3 (Fig. 1D). We analyzed the subcellular distributions of the predicted HRP59 protein isoforms by expressing cDNAs that encoded V5-tagged versions of HRP59-1 and HRP59-2 in S2 cells, preparing nuclear and cytoplasmic extracts, and analyzing the presence of each protein in the extracts by Western blotting (Fig. 1E). HRP59-1 was predominantly nuclear, as expected from our previous study (19), while HRP59-2 was excluded from the nucleus.

In summary, the results reported above show that the hrp59 pre-mRNA is alternatively spliced in S2 cells. One of the resulting hrp59 mRNAs, hrp59-1, has all the features of a functional mRNA and encodes a protein that is transported into the nucleus. The hrp59-2 mRNA, in contrast, appears to be a non-functional mRNA that is either degraded by NMD or translated into a truncated protein that accumulates in the cytoplasm.

The Levels of hrp59 mRNA Are Regulated in S2 Cells—Previous studies have shown that HRP59 binds preferentially to a subset of transcripts that includes the hrp59 mRNA itself (19). This observation suggests that there is a feedback mechanism that regulates the production of HRP59. We analyzed how the expression of the hrp59 gene is regulated by overexpressing HRP59-1 in S2 cells. We constructed a S2 cell line stably transfected with a plasmid that expresses the hrp59-1 cDNA under the control of an inducible metallothionein promoter (pMT-59). We constructed in parallel one control cell line transfected with a plasmid that expresses the dSF2/ASF (pMT-dSF2/ASF) and one transfected with the parental vector plasmid (pMT) (Fig. 2). Induction of the metallothionein promoter led to significant expression of HRP59-1 and dSF2/ASF, as shown by Western blotting using an anti-V5 antibody (Fig. 2A). We analyzed the effects of HRP59-1 overexpression on the levels of endogenous hrp59 pre-mRNA (2B) and mRNA (2C) by semiquantitative RT-PCR. The conditions used for PCR amplification were optimized to avoid saturation of the PCR reactions. Control reactions in the absence of reverse transcriptase were carried out in parallel and were negative (data not shown). Overexpression of HRP59-1 from the pMT-59 construct in stably transfected S2 cells caused a 3-fold increase of hrp59 pre-mRNA (Fig. 2B). In contrast, overexpression of dSF2/ASF
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FIGURE 2. HRP59 regulates the levels of its own mRNA. A, Western blot analysis of S2 cells expressing V5-tagged HRP59-1 and dSF2/ASF (control) before and after induction with 500 μM cupric sulfate. The blots were probed with a monoclonal anti-V5 antibody. B, RT-PCR analysis of hrp59 pre-mRNA levels in non-transfected S2 cells and in cells expressing HRP59-1 (pMT-59), dSF2/ASF (pMT-ASF), and pMT expression vectors. All cell lines were treated with 500 μM cupric sulfate for 48 h before RNA purification and RT-PCR analysis. The hrp59 pre-mRNA was amplified using a forward primer complementary to sequences in intron 3. The reverse primer was complementary to sequences in exon 4. C, RT-PCR analysis of endogenous hrp59 mRNA levels in S2 cells transfected as above. We ensured selective amplification of the endogenous hrp59 transcripts by using a forward primer that was complementary to a 5′-UTR sequence that is not present in pMT-derived transcripts. The reverse primer was complementary to sequences in exon 4. Bands corresponding to the abundant hrp59-1 and to the minor hrp59-2 transcripts are indicated. 18S rRNA in the same samples was analyzed in parallel and used for normalization. The histograms in B and C show the abundance of hrp59 pre-mRNA and hrp59-1 mRNA, respectively, relative to 18S rRNA. Data are presented as mean ± S.D. from three independent experiments.

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FIGURE 3. HRP59 regulates the splicing of its own pre-mRNA. A, depletion of HRP59 by RNAi in S2 cells shown by RT-PCR analysis of endogenous hrp59 transcripts. For RNAi, the S2 cells were treated twice with 20 μg of either GFP dsRNA (lane 2) or hrp59 dsRNA (lane 3). Cells without any dsRNA were also processed in parallel as controls (lane 1). Total RNA was isolated 96 h after the first addition of dsRNAs, and hrp59 transcript levels were analyzed by RT-PCR. The figure shows the exon structure of the hrp59 mRNA, and the arrows indicate the relative positions of the PCR primers used to detect the alternatively spliced transcripts. Black lines on the top of the diagram indicate the two regions targeted by the dsRNA. B, immunoblot analysis of HRP59 and dSF2/ASF proteins after RNAi. The lower panel shows anti-α-tubulin, used as a loading control. C, RT-PCR analysis of hrp59 transcripts derived from a full-length minigene expressed in S2 cells. The structure of the hrp59 minigene is shown, and the arrows indicate the locations and 5′-3′ orientations of the primers used in the RT-PCR assays. The dark boxes at both ends of the minigene correspond to the UTRs. Closed circles on exons 2 and 4 indicate the occurrence of the GAR motif. The structures of the spliced transcripts, based on sequence determinations of purified PCR bands, are also indicated. Note that RNAi-mediated knockdown of HRP59 results in the loss of the hrp59-2 transcript.

A reverse primer that was complementary to sequences in exon 4. Overexpression of HRP59-1 from the pMT-59 construct resulted in a 3-fold reduction in the levels of endogenous hrp59 mRNAs (Fig. 2C).

In summary, these results point to the existence of a feedback mechanism by which the HRP59-1 protein inhibits the splicing of the hrp59 pre-mRNA.

HRP59 Depletion Promotes the Formation of the hrp59-1 mRNA—To further analyze the effect of HRP59-1 on the alternative splicing of the hrp59 pre-mRNA, we silenced the expression of HRP59-1 in S2 cells by RNAi and analyzed the splicing of an hrp59 pre-mRNA expressed from a minigene after HRP59-1 depletion. We used a mixture of two dsRNAs corresponding to two different segments of the hrp59 mRNA (bars in Fig. 3A) for efficient depletion. The levels of endogenous hrp59 transcripts were drastically reduced after 4 days of RNAi treatment (Fig. 3A). The levels of HRP59 protein were also significantly reduced as shown by Western blot analysis (Fig. 3B). GFP and dSF2/ASF dsRNAs were used in parallel to assess the specificity of the depletion effects. After 4 days of RNAi treatment, the S2 cells were transiently transfected with a minigene that carried a

did not produce significant changes in the hrp59 pre-mRNA levels. We used 18S rRNA as an internal reference to monitor the efficiencies of total RNA purification and cDNA synthesis in the different samples. Quantification of the intensity of the PCR bands from three independent experiments showed that the effects were significant and specific for pMT-59. The detection of hrp59 pre-mRNA was carried out using a forward PCR primer that was located in intron 3 of the hrp59 pre-mRNA and that could not amplify the transcripts derived from the pMT-59 construct.

The endogenous hrp59 mRNAs (Fig. 2C) were detected exclusively using a forward primer that was complementary to the 5′ untranslated region of the hrp59 mRNA, which is not present in transcripts derived from the pMT-59 construct, and
synthetic hrp59 gene under the control of a constitutive promoter. This minigene included the genomic hrp59 sequences that lay between the start and the stop codons, including the introns (Fig. 3C). The 5′-UTR and 3′-UTR sequences of the synthetic hrp59 gene were those of the parental pAc5 vector. Primer pairs were designed to amplify both hrp59 splice variants in the same PCR reaction, as indicated in Fig. 3C. The forward primer was complementary to the 5′-UTR of the pAc5 vector, to ensure that only the transcripts derived from the plasmid were amplified. Two different hrp59 transcripts were amplified under these conditions (lane 1 in Fig. 3C), and sequencing of the PCR products confirmed that these two transcripts differed in the inclusion of exon 3. Interestingly, the hrp59-2 transcript was not detectable in HRP59-depleted cells (lane 4 in Fig. 3C). This effect was reproducible and specific. Both hrp59-1 and hrp59-2 mRNAs could be amplified in mock-treated cells (lane 1 in Fig. 3C) and in cells treated with dsRNA for either GFP or dSF2/ASF (lanes 2 and 3, respectively, in Fig. 3C). The same results were obtained when each of the two dsRNAs that target different regions of hrp59 (bars in Fig. 3A) were tested separately (data not shown).

In summary, these observations support the conclusion that HRP59 regulates the splicing of its own pre-mRNA and favors, directly or indirectly, the exclusion of exon 3 (see “Discussion”).

**HRP59 Regulates the Splicing of Other Transcripts**—We have previously identified several mRNAs that are preferentially associated with HRP59-1, one of which is the hrp59 mRNA itself. We have shown that most of these preferred mRNAs contain a common purine-rich sequence that resembles an exonic splicing enhancer of the (GAR)n type (19). HRP59-1 can regulate the splicing of its own pre-mRNA, as described above, and this suggests that HRP59-1 can regulate the splicing of other pre-mRNAs as well. The purine-rich sequence found in transcripts bound to HRP59-1 was highly enriched in some exons of the upheld (up) mRNA, also known as troponin T. Therefore we sought to determine whether HRP59-1 depletion has any effect on the splicing of the up pre-mRNA. The up pre-mRNA is ~8 kb long and contains 12 exons that are alternatively spliced into at least four different isoforms (28, 29). Due to the low levels of up expression in S2 cells, we designed a minigene called up-10. The up-10 construct contains the alternatively spliced exons 10A and B, and exon 11, which contains several copies of the purine-rich motif (Fig. 4). We expressed this minigene in transiently transfected S2 cells, and we analyzed by RT-PCR the splicing of the resulting pre-mRNAs. Sequencing of the PCR products showed that the two introns present in the up-10 pre-mRNA are efficiently spliced in control S2 cells (lane 1 in Fig. 4B). However, depletion of HRP59-1 by RNAi prevented up-10 pre-mRNA splicing (lane 4 in Fig. 4B). The only spliced transcript detected in the HRP59-1-depleted cells was a shorter, low abundance transcript that arose as a result of activation of a cryptic splice site in exon 11. From this we conclude that the levels of HRP59-1 affect the splicing of pre-mRNAs other than the hrp59 pre-mRNA itself. These results suggest that HRP59-1 is required for the proper splicing of the up pre-mRNA also under physiological conditions.

**DISCUSSION**

Our results show that the hrp59 transcripts are alternatively spliced to generate two mRNAs that differ in the presence or absence of exon 3. Most hrp59 mRNAs in Drosophila S2 cells contain exon 3 and code for a functional RNA-binding protein. Exon 3 exclusion results in a shift in the ORF that generates a PTC located 1500 nucleotides upstream of the last exon-exon boundary. We show that the PTC-containing transcripts are degraded by the nonsense-mediated mRNA decay pathway.

The existence of detectable levels of hrp59-2 mRNA suggests that the HRP59-2 isoform is synthesized in S2 cells. However, its functional significance is unclear. HRP59-1 is a predominantly nuclear protein that associates co-transcriptionally with a subset of mRNAs. In contrast, the HRP59-2 isoform is not imported into the nucleus. HRP59-2 has no obvious nuclear localization signals, and its nuclear import probably requires interacting proteins. HRP59-2 lacks two RNA-binding motifs (RRM 2 and RRM 3) and a glycine-rich region that is located between RRM 2 and RRM 3. Several studies have shown that RRMs and glycine-rich regions support important functions such as protein location, RNA-binding, protein self-association, and splicing regulation (30–32). The fact that HRP59-2 lacks these important domains suggests that this truncated HRP59 isoform is not functional. However, our present results do not exclude a cytoplasmic role for HRP59-2.

We demonstrate that HRP59-1 regulates the alternative splicing of the hrp59 pre-mRNA. In S2 cells, the inclusion of exon 3 appears to be more efficient than its exclusion, based on the observation that the hrp59-1 is the predominant hrp59 mRNA at steady state. Overexpression of HRP59-1 results in accumulation of hrp59 pre-mRNA with a concomitant reduction in the levels of spliced hrp59 mRNAs. These observations
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FIGURE 5. A model for the autoregulation of HRP59 expression. The hrp59 gene is alternatively spliced to generate two different mRNAs. Most hrp59 transcripts contain exon 3 and code for the functional nucleoplasmic HRP59-1 protein. Increased levels of HRP59-1 protein inhibit the production of hrp59-1 mRNA. Under these conditions the hrp59-2 mRNA can still be produced. The hrp59-2 transcript, however, contains a PTC and is kept at low levels by NMD. In this way, the HRP59-1 protein reduces the abundance of productive transcripts and hence the rate of protein production.

support a model in which HRP59 is a negative regulator of pre-mRNA splicing. We have also shown that depletion of HRP59 by RNAi favors the inclusion of exon 3. Two possible mechanisms can be envisioned to explain this observation: HRP59 either inhibits inclusion of exon 3 or facilitates exclusion of exon 3. The results from the overexpression experiment, however, are in conflict with HRP59 acting as a positive regulator. Therefore we favor a model in which HRP59 inhibits the inclusion of exon 3 (Fig. 5). According to this model, increased levels of HRP59-1 inhibit the formation of hrp59-1 mRNA, which in turn limits the production of HRP59-1 protein. Under these conditions, splicing leading to the formation of hrp59-2 can still take place, but the hrp59-2 levels are kept low by NMD. When HRP59-1 is down-regulated, the repression on the synthesis of hrp59-1 is released, the inclusion of exon 3 is more efficient, and exon 3 skipping is disadvantaged. In this way, alternative splicing and NMD are employed in a negative feedback circuit to control the abundance of HRP59-1 protein. In summary, our findings add HRP59 to the list of reported splicing factors that regulate their own expression by regulated unproductive splicing and translation (RUST) (33).

A recent study (34) on the role of hnRNP proteins in modulation of alternative splicing has led to the proposal that some hnRNP proteins, such as hnRNP A/B and F/H, can bind to both ends of an intron and bring distant splice sites together. The existence of HRP59-1 binding sites located in both exon 2 and exon 4 of the hrp59 transcript suggests that a similar model could apply here. However, the accumulation of hrp59 pre-mRNA transcript observed after HRP59 overexpression strongly suggests HRP59 acts as a negative regulator. For these reasons, we favor a model in which HRP59-1 acts by preventing the interaction of other splicing factors with the transcript.

Several authors have proposed a model in which the ratio of SF2/ASF-type SR proteins to A/B-type hnRNP s plays a role in regulating alternative splicing through antagonistic effects of the two protein types on splice site selection (reviewed in Ref. 35). The HRP59-binding motif is similar to the exonic splicing enhancer recognized by the human SF2/ASF (23), and we thus expected the insect SF2/ASF ortholog to compete with HRP59 for splicing regulation. However, neither SF2/ASF depletion nor overexpression affected the splicing of the minigenes tested. We suggest two possible explanations: either there is no competition for the same kind of binding sites, or other SR proteins can perform the required function.

We analyzed the splicing of the up-10 minigene in S2 cells and showed that depletion of HRP59-1 causes a drastic reduction of up-10 splicing. The up-10 is a synthetic transcript that may lack important regulatory sequences, and from our present experiment we cannot draw conclusions about the molecular mechanisms that lie behind the observed effects. However, our results indicate that the levels of HRP59-1 protein are important for the splicing activity of the cell and can affect the splicing of transcripts other than the hrp59 pre-mRNA.

In a previous study, we identified additional transcripts that bind HRP59 in S2 cells (19). Most of the identified transcripts contain introns, but not all of them are alternatively spliced, and a few of them are intronless. The activity of HRP59 in splicing regulation is likely to affect both general and alternative splicing. Moreover, HRP59-1 may also act by preventing splicing at cryptic or undesired splice sites. This possibility is supported by the observation that splicing of the up-10 transcript occurs at a new splice site when the levels of HRP59-1 are experimentally reduced.

In summary, our results show that the levels of HRP59 affect splice site selection and that HRP59 regulates its own expression by controlling the alternative splicing of its own pre-mRNA. We propose that this autoregulatory function serves as negative feedback control of HRP59 activity. Such a mechanism would ensure a constant level of HRP59 protein, which is important for the homeostasis of the splicing environment.

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