

Minimal functional domains of paralogues hnRNP L and hnRNP LL exhibit mechanistic differences in exonic splicing repression

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Understanding functional distinctions between related splicing regulatory proteins is critical to deciphering tissue-specific control of alternative splicing. The hnRNP (heterogeneous nuclear ribonucleoprotein) L and hnRNP LL (hnRNP L-like) proteins are paralogues that have overlapping, but distinct, expression patterns and functional consequences. These two proteins share high sequence similarity in their RRM (RNA-recognition motifs), but diverge in regions outside of the RRMs. In the present study, we use an MS2-tethering assay to delineate the minimal domains of hnRNP L and hnRNP LL which are required for repressing exon inclusion. We demonstrate that for both proteins, regions outside the RRMs, the N-terminal region, and a linker sequence between RRMs 2 and 3, are necessary for exon repression, but are only sufficient for repression in the case of hnRNP LL. In addition,

both proteins require at least one RRM for maximal repression. Notably, we demonstrate that the region encompassing RRMs 1 and 2 of hnRNP LL imparts a second silencing activity not observed for hnRNP L. This additional functional component of hnRNP LL is consistent with the fact that the full-length hnRNP LL has a greater silencing activity than hnRNP L. Thus the results of the present study provide important insight into the functional and mechanistic variations that can exist between two highly related hnRNP proteins.

Key words: alternative splicing, heterogeneous nuclear ribonucleoprotein L (hnRNP L), heterogeneous nuclear ribonucleoprotein LL (hnRNP LL), MS2 tethering, spliceosome regulation.

INTRODUCTION

The hnRNP (heterogeneous nuclear protein) family of RNA-binding proteins are a set of highly diverse proteins with respect to both structure and function. All hnRNP proteins contain at least one known RNA-binding motif, typically of the RRM (RNA-recognition motif) or KH (K homology) type, and bind to both nascent transcripts and mature mRNAs to regulate everything from splicing to translation [1–3]. In some cases the binding of the hnRNP to substrates is sufficient for function, such as in instances in which binding of the hnRNP occludes a splice site or another required sequence [3]. However, such steric-hindrance models account for only a subset of hnRNP function [4]. Importantly, in addition to their RNA-binding domains, most hnRNPs contain regions of low-complexity sequence or other spacer regions of unknown function. The contribution of such additional sequences to hnRNP splicing regulatory activity is largely unexplored.

Although most hnRNPs are thought to function ubiquitously, some instances of cell-type specific expression have been identified. Most notably, several ‘ubiquitous’ hnRNPs have paralogues that are expressed in a more tissue-restricted manner. Well-studied examples include nPTB [neural PTB (polypyrimidine tract-binding protein)], a neuronal-biased paralogue of PTB [5,6], and hnRNP LL (hnRNP L-like), a paralogue of hnRNP L that is expressed preferentially in activated T-cells and plasma B-cells [7–9]. Consistent with the high degree of sequence similarity, particularly within their RNA-binding domains, hnRNP paralogues such as PTB/nPTB and hnRNP L/LL show similar specificity for RNA substrate recognition. Notably, however, several lines of evidence suggest that the paralogues have differential function even when bound to the

same substrate. Moreover, the functional distinction between these ubiquitous and tissue-specific paralogues correlates with tissue-specific differences in gene expression. For example, PTB represses use of the neural-specific N1 exon of the *n-Src* gene in most tissues, whereas substitution of PTB with nPTB relieves repression of this exon in neurons [5]. Similarly, we and others have shown that hnRNP L and hnRNP LL both bind to the same regulatory element in exon 4 of the CD45 gene [7,10], but expression of hnRNP LL in activated T-cells induces repression more than is conferred by a corresponding change in hnRNP L expression [7–9]. Therefore understanding the molecular basis of the differential function of hnRNP paralogues is essential to more fully understanding the determinants of tissue-specific regulation of splicing.

In the present study we use an MS2-tethering approach and a well-characterized substrate of hnRNP L and hnRNP LL function, to define the minimal domain requirements for hnRNP-mediated splicing repression. Both proteins contain four highly conserved RRM domains, an N-terminal sequence that for hnRNP L is rich in glycine and a sequence linking RRMs 2 and 3 that is enriched in proline, especially in the case of hnRNP L (Figure 1). We found that for both hnRNP L and LL, sequences outside of their RRMs are necessary to mediate splicing repression. These repression domains include the N-terminal and linker regions that differ most significantly between hnRNP L and LL. We also demonstrate that sequences encompassing at least one RRM is required for maximal repression by each protein, even when RNA binding is mediated through the heterologous MS2-hairpin interaction. Finally, we identify a region of hnRNP LL that is responsible for an additional exon repressive activity that is not observed within hnRNP L. This additional hnRNP LL-specific activity provides

Abbreviations used: HEK, human embryonic kidney; hnRNP, heterogeneous nuclear protein; hnRNP LL, hnRNP L-like; PTB, polypyrimidine tract-binding protein; nPTB, neural PTB; snRNP, small nuclear ribonucleoprotein.

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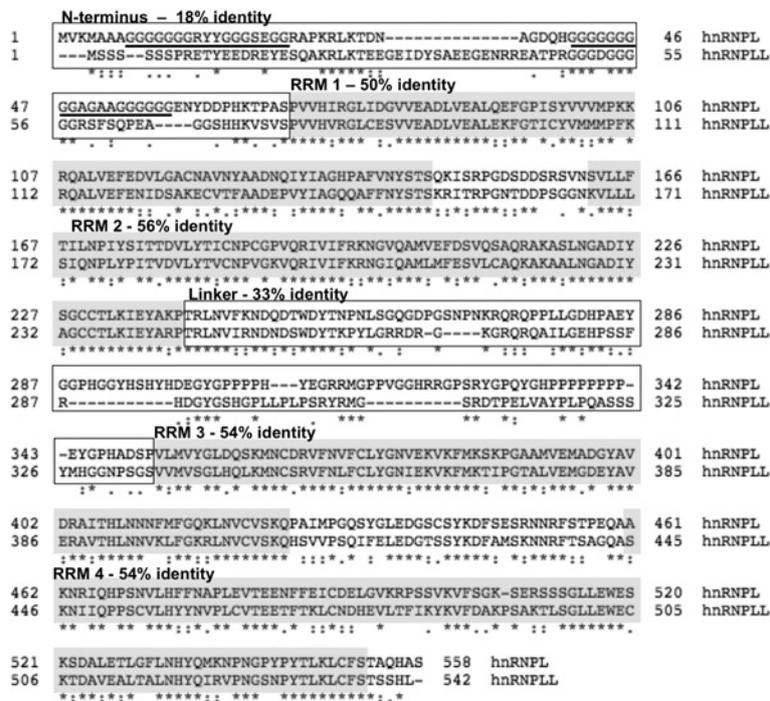


Figure 1 Sequence, domain structure and conservation of hnRNP L and hnRNP LL

Amino acid sequence of hnRNP L (upper) and hnRNP LL (lower). The domains, and the boundaries thereof, used in the present study are indicated above the sequences. The percentage amino acid identity for each domain is given above the sequences, asterisks indicate positions which have a single fully conserved residue, a colon indicates conservation between groups of strongly similar properties (scoring >0.5 in the Gonnet PAM 250 matrix), and a full stop indicates conservation between groups of weakly similar properties (scoring <0.5 in the Gonnet PAM 250 matrix). N-terminal and linker regions are indicated by a box, the glycine-rich region in hnRNP L is underlined and RRM3 are highlighted in grey.

an explanation for the functional distinctions between hnRNP L and hnRNP LL, which have been observed in previous studies.

EXPERIMENTAL

Plasmids

The CD24MS2 minigene was constructed by replacing the naturally occurring ESS (exonic splicing silencer) element with a sequence encoding a single copy of the MS2 bacteriophage stem-loop RNA. The various domain deletion derivatives of hnRNP L and hnRNP LL were created using overlap PCR mutagenesis containing the BamHI and EcoRI sites, and cloned into similarly digested FLAG-MS2 constructs. The exact nucleotide and amino acid boundaries for each mutant are shown in Table 1. The GFP sequence is as described in Topp et al. [7]. The A1 and A1 + U1 plasmids used in Figure 6 are identical to the A and Q mutations respectively published previously [11].

Cell culture

HEK (human embryonic kidney)-293 cells were cultured in Dulbecco's modified Eagle's medium (Cellgro-Mediatech), supplemented with 10% fetal bovine serum (Gibco).

Transfection

For the MS2-tethering assay, HEK-293 cells were seeded on to six-well plates at 500 000 cells/well, and co-transfected with 800 ng of the CD24MS2 minigene and 1.5 μ g/well of plasmid construct encoding various derivatives of hnRNP L and hnRNP

LL fused to the MS2 RNA-binding domain and the FLAG epitope. All transfections were carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

RNA and protein analysis

HEK-293 cells were harvested 48 h post-transfection for RNA and protein analysis. RNA was isolated using RNA-Bee and semi-quantitative RT (reverse transcription)-PCR was conducted as described previously [7]. Nuclear proteins were extracted by lysing cells in buffer containing 10 mM Hepes, 60 mM KCl, 1 mM EDTA, 0.075% Nonidet P40 and protease inhibitor cocktail (Sigma). Cell lysates were centrifuged at 16000 g for 5 min and the nuclear pellet was resuspended in buffer containing 20 mM Tris/HCl, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol and protease inhibitor cocktail (Sigma). The pellet was incubated for 1 h on ice with repeated vortex-mixing and centrifuged at 16000 g for 10 min. The supernatant was mixed with 5 \times SDS sample buffer, resolved by SDS/PAGE (10% gel) and immunoblotted using anti-FLAG (Sigma) or anti-(hnRNP A1) (Abcam) antibodies.

RESULTS

hnRNP LL has greater repressive activity than hnRNP L when tethered in a similar manner to an exon

CD45 exon 4 is the best characterized example of splicing repression mediated by hnRNP L and hnRNP LL. Previous studies have demonstrated that (i) hnRNP L and hnRNP LL bind to a similar region of CD45 exon 4 [7,10]; (ii) hnRNP L and hnRNP

Table 1 Breakpoints of the domains used in the fusion proteins in the present study

hnRNP L derivative	Amino acids encoded	Amino acid sequence	Nucleotide sequence
ΔG	59–558	ENY...HAS	GAGAACTAC...CACGCCTCC
ΔR1	1–70 + 146–558	MVK...PAS + QKI...HAS	ATGGTGAAG...CCTGCCTCC + CAGAAGATC...CACGCCTCC
ΔR2	1–161 + 240–558	MVK...SVN + TRL...HAS	ATGGTGAAG...AGCGTGAAC + ACACGCTTG...CACGCCTCC
ΔLink	1–239 + 352–558	MVK...AKP + VLM...HAS	ATGGTGAAG...GCAAAGCCT + GTGCTCATG...CACGCCTCC
Δ34	1–351	MVK...DSP	ATGGTGAAG...GACAGCCCT
Gly	1–70	MVK...PAS	ATGGTGAAG...CCTGCCTCC
Link	240–351	TRL...DSP	ACACGCTTG...GACAGCCCT
G + Link	1–70 + 240–351	MVK...PAS + TRL...DSP	ATGGTGAAG...CCTGCCTCC + ACACGCTTG...GACAGCCCT
G1L	1–145 + 240–351	MVK...PAS + PVV...STS + TRL...DSP	ATGGTGAAG...CCTGCCTCC + CCAGTTGC...TCTACCAGC + ACACGCTTG...GACAGCCCT
G2L	1–70 + 162–239 + 240–351	MVK...PAS + SVL...AKP + TRL...DSP	ATGGTGAAG...CCTGCCTCC + AGTGTGCTT...GCAAAGCCT + ACACGCTTG...GACAGCCCT
G3L	1–70 + 352–425 + 240–351	MVK...PAS + VLM...SKQ + TRL...DSP	ATGGTGAAG...CCTGCCTCC + GTGCTCATG...TCCAAGCAG + ACACGCTTG...GACAGCCCT
G4L	1–70 + 461–552 + 240–351	MVK...PAS + AKN...CFS + TRL...DSP	ATGGTGAAG...CCTGCCTCC + GCCAAGAAC...TGTTTCTCC + ACACGCTTG...GACAGCCCT
G–GFP–Link	1–70 + GFP + 240–351	MVK...PAS + GFP + TRL...DSP	ATGGTGAAG...CCTGCCTCC + GFP + ACACGCTTG...GACAGCCCT
G–flex–Link	1–70 + Flexible linker + 240–351	MVK...PAS + (AAQAAQAAQ) ₂ AA + TRL...DSP	ATGGTGAAG...CCTGCCTCC + FLEXI + ACACGCTTG...GACAGCCCT
(b)			
hnRNP LL derivative	Amino acids encoded	Amino acid sequence	Nucleotide sequence
ΔN	76–542	PVV...SHL	CCCGTCGTC...TCCCATTTA
ΔLink	1–244 + 336–542	MSS...ARP + VVM...SHL	ATGTCCTCC...GCACGGCCA + GTTGTAATG...TCCCATTTA
ΔR34	1–335	MSS...SGS	ATGTCCTCC...TCTGGTTCA
ΔLink34	1–244	MSS...ARP	ATGTCCTCC...GCACGGCCA
LL-N	1–75	MSS...SVS	ATGTCCTCC...TCTGGTTCA
LL-Link	245–335	TRL...SGS	ACTCGTCTA...TCTGGTTCA
NLink	1–75 + 245–335	MSS...SVS + TRL...SGS	ATGTCCTCC...TCTGGTTCA + ACTCGTCTA...TCTGGTTCA
N1L	1–75 + 76–150 + 245–335	MSS...SVS + PVV...STS + TRL...SGS	ATGTCCTCC...TCTGGTTCA + CCCGTCGTC...TCTACAAGC + ACTCGTCTA...TCTGGTTCA
N2L	1–75 + 167–244 + 245–335	MSS...SVS + KVL...ARP + TRL...SGS	ATGTCCTCC...TCTGGTTCA + AAAGTCTT...GCACGGCCA + ACTCGTCTA...TCTGGTTCA

LL are not functionally redundant on this exon [7–9]; and (iii) repression of exon 4 by hnRNP L does not involve mere steric hindrance, rather hnRNP L recruits hnRNP A1 to induce an extended interaction between the exon and the spliceosomal U1 snRNP (small nuclear ribonucleoprotein) component to inhibit proper spliceosome assembly ([11]; see below). Therefore CD45 exon 4 is an optimal system in which to begin to better define the domain requirements for hnRNP L-mediated exon repression and to understand the functional differences between the paralogues hnRNP L and LL.

The viral coat protein MS2 is a small protein that binds with high affinity to a cognate RNA hairpin sequence [12]. This interaction is widely used to recruit proteins of interest to RNA substrates through the generation of chimaeric MS2 fusion proteins [13]. We have previously used such an MS2-tethering assay *in vitro* to demonstrate that hnRNP L contains silencing activity on CD45 exon 4 beyond that achieved simply through RNA binding [11,14]. As a first step towards comparing the silencing activities of hnRNP L and hnRNP LL, we adapted the MS2 assay for use in cells with both of the hnRNP L/LL paralogues.

cDNA encoding the full-length hnRNPL or hnRNPLL proteins were fused to sequences encoding a FLAG epitope and the MS2 coat protein (Figure 2a). This expression plasmid was co-expressed in HEK-293 cells with a plasmid encoding a minigene in which CD45 exon 4 carries a single copy of a sequence encoding the MS2 replicase stem-loop element in place of the normal

silencer sequence (Figure 2a, CD24MS2; [14]). A standard low-cycle RT-PCR assay was then used to quantify the splicing of RNA derived from the minigene in the presence of the MS2-fusion proteins or the MS2 protein alone as a control (Figure 2b; see the Experimental section).

Consistent with our previous *in vitro* results, the MS2–hnRNP L fusion protein caused a 30% increase in skipping of the exon containing the MS2-binding site when compared with cells transfected with the MS2 vector alone (Figure 2b, MS2 compared with MS2-L, 45% inclusion to 15% inclusion). As a control, a minigene containing the MS2 stem-loop element in the reverse orientation, which does not bind the MS2 protein, was largely unaffected by MS2–hnRNP L (Figure 2b, Rev). Transfection of increasing amounts of the MS2–hnRNP L-encoding vector beyond 500 ng of DNA did not cause any further increase in exonic repression activity despite increased protein expression (Figures 2c and 2d). Saturation of the assay at low protein expression levels allows us to assume that small variations in the expression levels of the various deletion mutants will not impact the observed level of repression, hence precluding a need to normalize exon inclusion to protein expression.

Similar to the repression by MS2-L, the MS2–hnRNP LL fusion protein also confers significant and specific repression of the MS2 hairpin-containing exon, even at the lowest level of protein expression tested (Figures 2b–2d, MS2-LL). Interestingly, we observe consistently greater repression with MS2–hnRNP LL relative with MS2–hnRNP L. Even at the least amount of

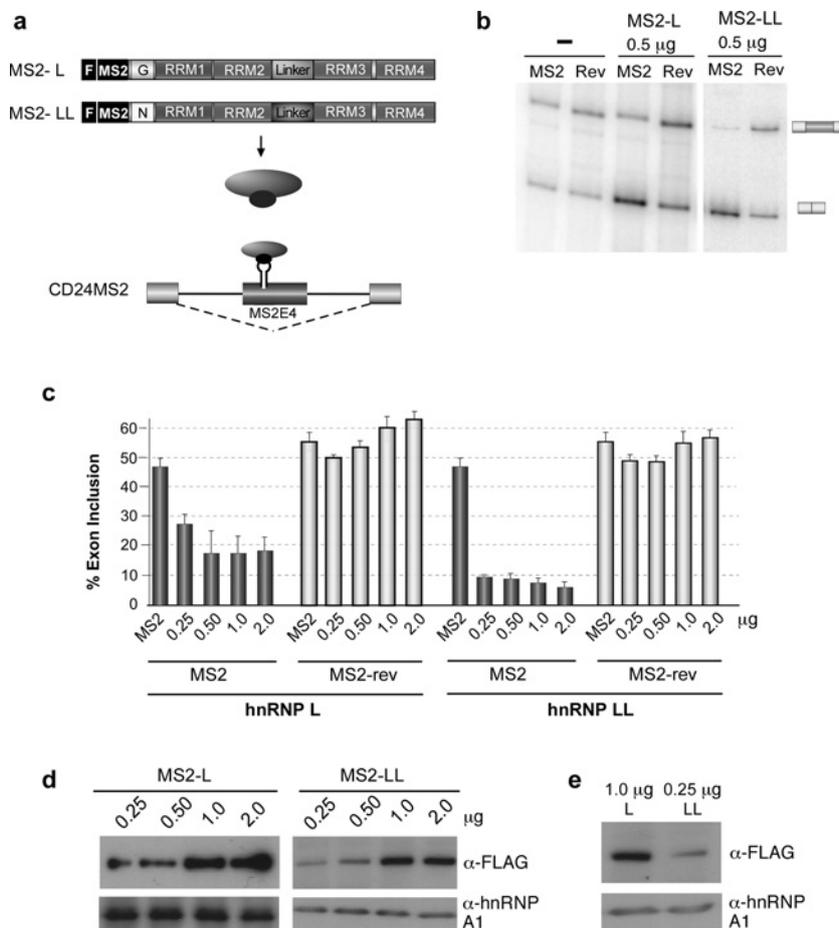


Figure 2 MS2-tethering assay recapitulates the exon repressive activity of hnRNP L and hnRNP LL

(a) Schematic diagram of hnRNP L and hnRNP LL fusions with an N-terminal FLAG tag (F) and coding sequence for the MS2 bacteriophage coat protein (MS2). The splicing reporter CD24MS2 minigene consists of CD45 exon 4 which carries a single copy of the MS2 stem-loop-binding site flanked by CD45 exons 3 and 7. For CD24MS2, the boxes denote exons, whereas thin lines denote introns. (b) RT-PCR analysis of CD24MS2 expression in the absence or presence of MS2-hnRNP L (MS2-L) or MS2-hnRNP LL (MS2-LL) co-transfected in HEK-293 cells. (c) Quantification of the RT-PCR assays shown in (b) performed over a range of co-transfected MS2-L or MS2-LL expression plasmids. Values graphed represent mean inclusion of MS2E4 exon (see a, central exon) calculated from biologic triplicates. Error bars represent S.E.M. (d) Immunoblot of nuclear lysates from the transfected cells of (c) using an anti-FLAG antibody (for MS2-L/MS2-LL) or an anti-(hnRNP A1) antibody to assess loading. (e) Nuclear lysates from cells transfected with given amounts of MS2-L (L) or MS2-LL (LL) cDNA, blotted together to demonstrate relative expression.

hnRNP LL protein used, repression is significantly greater than is observed at saturation with hnRNP L (Figures 2c and 2e, 8% compared with 16% inclusion, $P < 0.01$). This difference cannot be attributed to differences in RNA-binding affinity given that the binding of each of the fusion proteins is mediated by the same MS2 motif, but is consistent with the fact that increased expression of hnRNP LL in T-cells results in more repression of CD45 exon 4 than can be achieved by increasing expression of hnRNP L alone [7,8].

Identification of the minimal domains of hnRNP L necessary and sufficient for repressing exon inclusion

As a first step towards investigating the role of the various domains of hnRNP L in splicing repression function, we created a series of plasmid constructs that encode truncated forms of MS2-hnRNP L (Figure 3a). Repeating the splicing assay outlined in Figure 2, we find that that RRMs 3 and 4 are dispensable for the exonic repression activity of hnRNP L (Figures 3b and 3c, MS2 compared with $\Delta R34$). Moreover, individual removal of

either of RRM 1 or RRM 2 only reduces the repressive activity of the fusion protein by less than ~25% (Figures 3b and 3c, $\Delta R1$ and $\Delta R2$). Note that simultaneous deletion of RRMs 1 and 2 did not produce stable protein and thus these constructs were not assayed. By contrast, deletion of the glycine-rich domain reduced the hnRNP L splicing repression activity to 42% of that of the full-length protein, whereas deletion of the linker region reduces repression by over 80% {Figures 3b and 3c, ΔG and $\Delta Link$, relative percentage activity = $[(\%inclusion)_{MS2} - (\%inclusion)_{mutant}] / [(\%inclusion)_{MS2} - (\%inclusion)_{FL}]$ }. In all cases, we confirmed the localization of the MS2 fusion proteins in the nucleus (Figure 3c). Moreover, the ΔG and $\Delta Link$ constructs retained interaction with the known hnRNP L-interacting partner Prp19 (results not shown), suggesting that loss of splicing repression function was not due to gross misfolding of these truncated proteins.

We next tested whether the linker and/or the glycine-rich domain were sufficient for repressing exon inclusion. Neither the glycine-rich or the linker domain, singly or in combination, have more than 20% repressive activity when fused to MS2 (Figure 4, Gly, Link and G + Link). By contrast, the addition

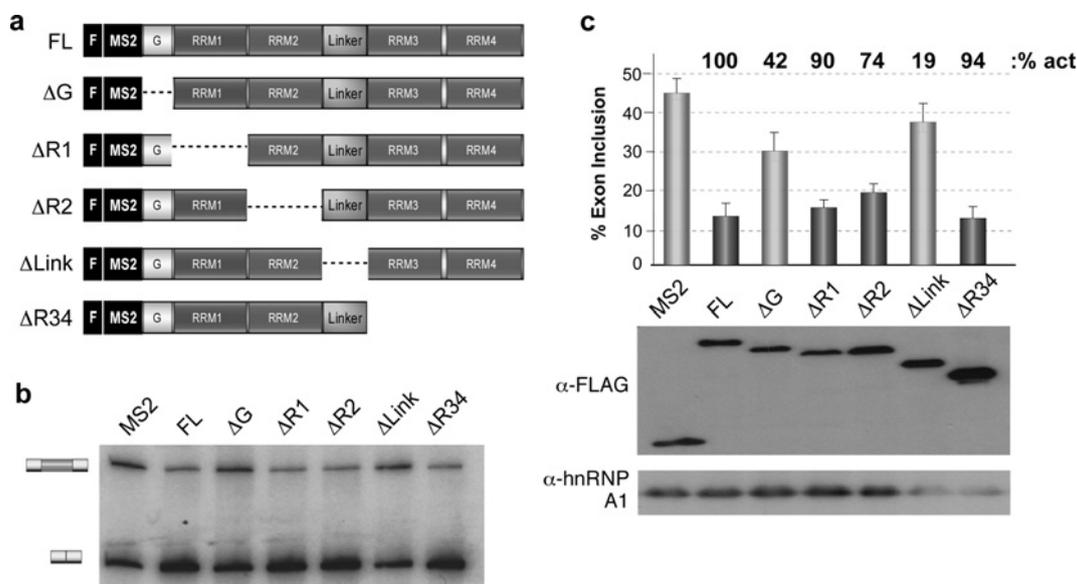


Figure 3 Multiple domains of hnRNP L are required for exonic repression

(a) Illustration of various domain deletion derivatives of hnRNP L. The dotted area in each construct denotes the domain that is deleted. (b) RT-PCR analysis of CD24MS2 splicing in the presence of each of the hnRNP L deletion derivatives. (c) Upper panel, histogram of the mean percentage inclusion (\pm S.E.M.) of MS2E4 from at least three independent replicates of RT-PCR as in (b). The percentage activity (% act) relative to full-length MS2-hnRNP L (as defined in the text) is given at the top of the histogram. Lighter bars indicate constructs that exhibit less than 50% of the repressive activity of full-length MS2-L. Lower panel, immunoblot of nuclear lysates from transfected cells as in (b and c) using an anti-FLAG antibody to determine the expression of MS2-L derivatives or an anti-(hnRNP A1) antibody as a loading control.

of RRM 3 or 4 between the glycine and linker domains restores repression to over 60% the extent observed with the full-length protein, whereas the addition of RRM 2 yields repression that is virtually indistinguishable from the wild-type (Figure 4, G2L, G3L and G4L). Strikingly, however, RRM 1 is not sufficient to confer any repression when substituted for any of the other RRMs, despite being similarly expressed and localized to the nucleus as the other RRM-containing fusions (Figures 4a and b, G1L). To ask if the requirement for RRMs 2–4 simply reflected a particular structural requirement we also fused the glycine-rich and linker regions to either GFP, a well-folded domain, or a flexible linker sequence (Figure 4a, G-GFP-Link and G-flex-Link). Interestingly, neither increasing (G-GFP-Link) nor decreasing (G-flex-Link) the structural constraints on the fusion protein restored splicing repressive activity to more than a third of that of the full-length protein (Figure 4b). Therefore we conclude that RRM 2, and to a lesser extent RRMs 3 and 4, each contain some specific activity that together with the glycine-rich and linker domain accounts for the splicing repressive activity of hnRNP L.

N-terminal and linker regions of hnRNP LL are sufficient for repression

We next investigated whether domains of hnRNP LL, analogous to those identified in hnRNP L, are similarly necessary and/or sufficient for repression. Consistent with hnRNP L, removal of the two C-terminal RRMs of hnRNP LL has no effect on the ability of the MS2-hnRNP LL fusion protein to repress inclusion of the MS2E4 exon (Figures 5a and 5b, Δ R34). However, further truncation of the linker sequence or specific deletion of the linker domain, reduces the repressive activity of the fusion protein by 66% (Figures 5a and 5b, Δ Link34 and Δ Link). Interestingly, specific deletion of the N-terminal sequence of hnRNP LL only reduces the repression efficiency by 30% (Figures 5a and 5b,

Δ N). Therefore the N-terminus of hnRNP LL contributes less to the overall repression of the full-length protein than does the glycine-rich N-terminus of hnRNP L.

Although the linker region of hnRNP LL is the only domain absolutely required for exon repression, this sequence only confers 49% of the maximal repression when fused alone to MS2 (Figures 5c and 5d, LL-Link). Notably, the N-terminal region of hnRNP LL also confers 49% of the maximal repression; however, the addition of the N-terminus to the linker region is sufficient to achieve 66% repression (Figures 5c and 5d, LL-N compared with NLink). Moreover, unlike what we observed for hnRNP L, the addition of RRMs between the N-terminal and linker sequences only increases marginally the repressive activity to this minimal construct (Figures 5c and 5d; N1L and N2L compared with NLink). Therefore the N-terminus and linker regions of hnRNP LL have more repressive activity than the corresponding domains of hnRNP L and the RRMs, at least individually, do not significantly contribute to repression.

hnRNP LL and hnRNP L repress exon inclusion through distinct mechanisms

Given the distinct domains of hnRNP L and hnRNP LL that are involved in exon repression, and the different extent of repression observed by these two proteins, we wanted to determine if these proteins function through the same or different mechanisms. Previously we have shown that hnRNP L represses CD45 exon 4 by recruiting hnRNP A1 to the 3'-end of the exon and inducing extended base-pair interactions between the U1 snRNA and the exon. The interaction of hnRNP A1 and the U1 snRNA with the exon then block the progression of spliceosome assembly to prevent exon inclusion ([11]; Figure 6a). A hallmark of this mechanism is that the repression is significantly reduced by mutations in the hnRNP A1-binding site (A1) or mutations that disrupt both the binding of hnRNP A1 and base-pairing of U1

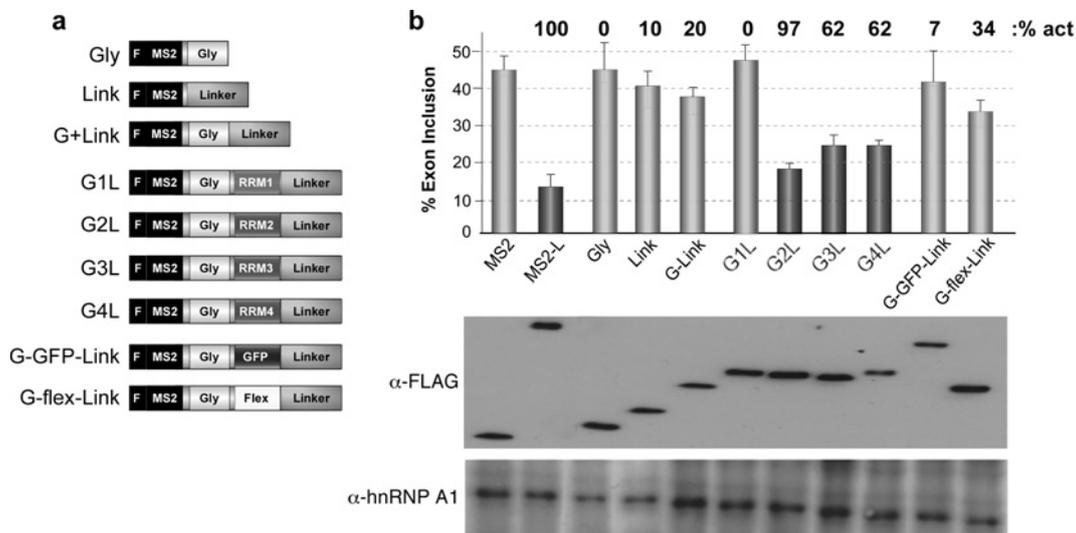


Figure 4 Minimal hnRNP L-derived repression requires the glycine-rich and linker domains with either RRM 2, 3 or 4

(a) Illustration of constructs encoding various domain combinations of hnRNP L. (b) Upper panel, histogram of the mean percentage inclusion (\pm S.E.M.) of MS2E4 from at least three independent replicates of RT-PCR using the glycine-Linker domain set from (a). The percentage activity (% act) relative to full-length MS2-hnRNP L (as defined in the text) is given at the top of the histogram. Lighter bars indicate constructs that exhibit less than 50% of the repressive activity of full-length MS2-L. Lower panel, immunoblot of nuclear lysates from transfected cells using an anti-FLAG antibody to determine the expression of MS2-L derivatives or an anti-(hnRNP A1) antibody as a loading control.

snRNA (A1 + U1) ([11]; Figure 6a). We note that even in the absence of hnRNP L (or hnRNP LL) the A1 and A1 + U1 mutations reduce exon skipping as hnRNP A1 can bind weakly to its cognate site without the assistance of hnRNP L ([11]; Figure 6b, MS2).

Consistent with the above model of the repression of exon 4, the A1 + U1 mutation reduces the repressive activity of MS2-hnRNP L by greater than 2-fold (Figure 6b, MS2-L). We note that the A1 mutation only weakens the repression of MS2-hnRNP L by \sim 15% (63 to 54% repression), presumably since the high-affinity binding of MS2-hnRNP L is able to compensate somewhat for the loss of A1 affinity induced by the single point mutation. However, in striking contrast to what is observed with MS2-hnRNP L, 98% or 88% of the repression by MS2-LL is retained in the A1 and A1 + U1 mutants respectively (Figure 6b, MS2-LL). Indeed, even in the presence of the A1 + U1 mutant, MS2-LL represses exon inclusion to a greater extent as MS2-L is able to maximally achieve on the wild-type exon (77% compared with 63%).

To further probe the differential activities of the individual domains of hnRNP L and LL, we next investigated the activity of the minimal functional versions of hnRNPL and LL. Surprisingly, the repressive activity of the G2L and NLink versions of hnRNP L and LL respectively, exhibit similar or greater sensitivity to the A1 and A1 + U1 mutants than MS2-hnRNP L. Specifically, repression by both G2L of hnRNP L and NLink of hnRNP LL is reduced by more than 75% in the absence of A1 and U1 association with the exon (Figure 6b, WT compared with A1 + U1). Together with the similar extent of repression of the wild-type exon conferred by these mutants (\sim 60% repression for both G2L and NLink on wild-type MS2E4, Figure 6b), this suggests that hnRNP L and hnRNP LL function by an overlapping mechanism that involves recruitment of hnRNP A1 and the U1 snRNA to the exon (see below for further discussion). Strikingly, however, the Δ R34 version of hnRNP LL both achieves greater absolute repression than LL(NLink) (90% compared with 55%). Moreover LL(Δ R34) exhibits 67% repression even in the A1 + U1 mutant (74% of that observed

for the wild-type exon). Therefore we conclude that the region of hnRNP LL encompassing RRM1 and 2 contains a second repressive activity that robustly represses splicing even in the absence of efficient U1 snRNA interactions with the exon.

DISCUSSION

The hnRNP family of proteins encompasses a large number of structurally and functionally diverse RNA-binding proteins which play a widespread role in regulating alternative splicing. Interestingly, in some cases multiple divergent hnRNPs function in a redundant manner with each other [15], whereas in other instances individual hnRNPs have been shown to have distinct functions on a given gene target. Most striking, and unexplained, are differential functions that have been shown to exist between close paralogues such as PTB/nPTB and hnRNP L/hnRNP LL. Such paralogues have a high sequence identity and RNA-binding specificity, yet often exhibit non-redundant activity.

In the present study we identify the minimal protein domains required for exon repression by hnRNP L and hnRNP LL. Both proteins share the common feature that their N-terminus and the peptide region linking RRM2 and 3 (i.e. the Linker region) are both required for repression. Moreover, in each case at least some additional sequence, encompassing one or more RRMs, is required for the full repressive activity, although the absolute requirement for an RRM differs. Specifically, the NLink construct of hnRNP LL, which lacks any RRM sequence, contains as much exon repressive activity as does the full length hnRNP L, whereas the corresponding G-Link construct of hnRNP L has little ability to confer exon skipping. Interestingly, this result demonstrates that the glycine- and proline-rich characteristics of the N-terminus and linker regions respectively of hnRNP L do not confer efficient repressive activity as has been shown for other hnRNPs [16–18]. Rather the less uniform sequence of the N-terminus and linker regions of hnRNP LL has greater activity. These results underscore how relatively small or unappreciated sequence differences between paralogues can have profound functional consequences which are difficult to predict a priori.

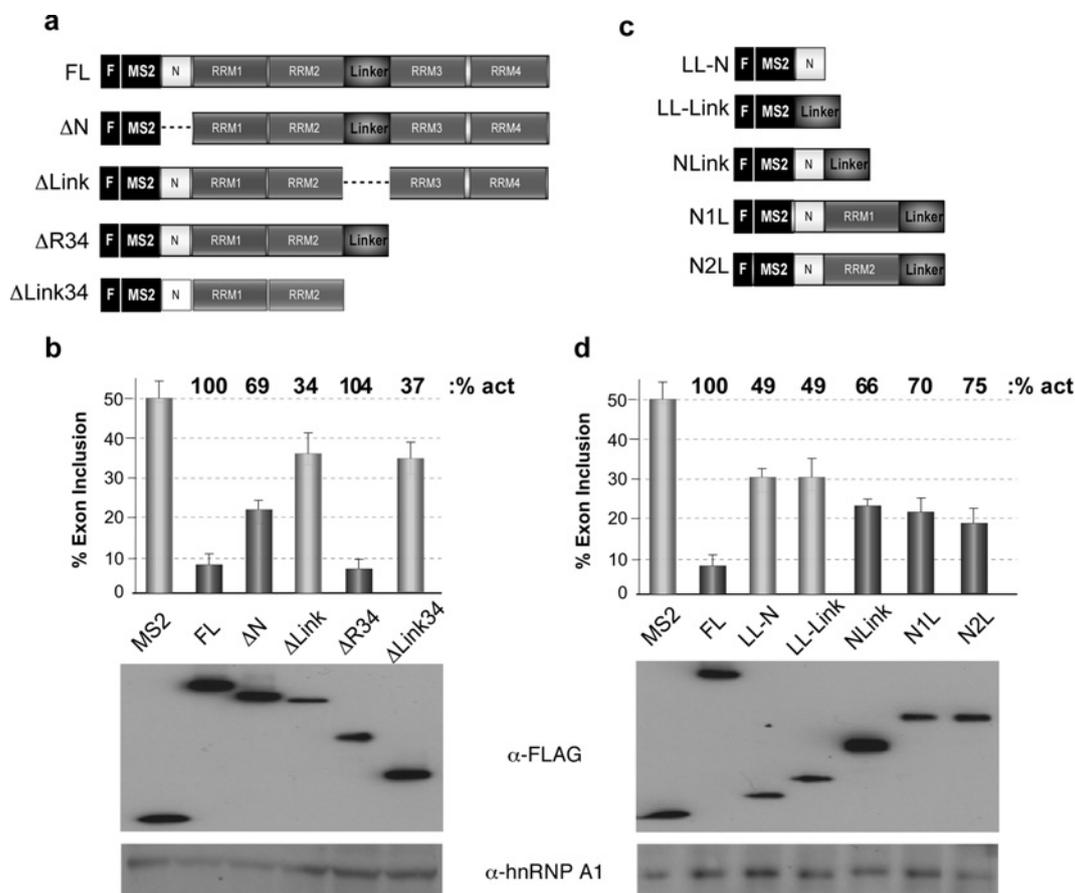


Figure 5 Multiple domains of hnRNP LL are required for exonic repression

(a) Illustration of plasmid constructs encoding various domains deletion derivatives of hnRNP LL. The dotted area in each construct denotes the domain that is deleted. (b) Upper panel, histogram of the mean percentage inclusion (\pm S.E.M.) of MS2E4 from at least three independent replicates of RT-PCR as in Figure 3(B). The percentage activity (% act) relative to full-length MS2-hnRNP L (as defined in the text) is given at the top of the histogram. Lighter bars indicate constructs that exhibit less than 50% of the repressive activity of full-length MS2-L. Lower panel, immunoblot of nuclear lysate from transfections with an anti-FLAG antibody to determine the expression of MS2-LL derivatives or an anti-(hnRNP A1) antibody as a loading control. (c) Schematic diagram of minimal hnRNP LL domain fusion proteins. (d) Upper panel, histogram of the mean percentage inclusion (\pm S.E.M.) of MS2E4 from at least three independent replicates of RT-PCR as in Figure 3(B). Lower panel, immunoblot of nuclear lysate from transfections with an anti-FLAG antibody to determine expression of MS2-LL derivatives or an anti-(hnRNP A1) antibody as a loading control.

Although a strict requirement for an RRM to achieve repression is only observed for hnRNP L, the presence of sequences encompassing RRM1 and 2 greatly stimulates the repressive activity of hnRNP LL. In the experimental set up used in the present study, the high-affinity MS2 RNA-binding protein was used to recruit all of the constructs to the RNA; therefore, it is unlikely that influence of an RRM-containing sequence on repression activity was due to constraints for substrate binding. Rather we assume that the RRM1, or immediate flanking regions, are involved in protein-protein interactions which are required for splicing repression, although we also cannot rule out that the RRM1 of hnRNP L or LL are required to contact some RNA component of the spliceosome to affect repression. Importantly, precedence for both models exist in that RRM 2 of the related hnRNP PTB is required for exon repression by PTB and as has been shown to interact with both the co-repressor protein Raver1 (ribonucleoprotein, PTB-binding 1) as well as with a portion of the U1 snRNA to block spliceosome assembly [19–21].

Finally, we note that the activity conferred by the RRM1 on hnRNP L and LL is specific for certain RRM1. For hnRNP L, only RRM1, 2, 3 and 4 promote repression, whereas the first RRM lacks activity. A basic sequence alignment does not reveal any obvious feature that distinguishes RRM1–4 from RRM 1, neither does

this appear to be a simple requirement for more or less structure (see Figure 4). However, future experiments probing specific differences between RRM 1 and 2–4 should be informative in gaining a detailed molecular understanding of the requirements for repression.

Similarly, the region encompassing RRM1 and 2 of hnRNP LL hold particular interest in that this sequence appears to contain a hyper-repressive activity. Most importantly, this extra activity is insensitive to mutations that disrupt repression by hnRNP L, implying that this peptide blocks exon inclusion by a distinct mechanism. Since hnRNP LL(NLink) has activity that is similar to full-length hnRNP L in both degree of repression and sensitivity to the A1 and U1 mutations, we conclude that the hyper-repression observed with the full-length or Δ R34 versions of hnRNP LL is due to a ‘double hit’ in which a second activity conferred by RRM1 and 2 of hnRNP LL is layered on top of the A1/U1 snRNA-remodelling activity conferred by the NLink domains of hnRNP LL or hnRNP L. It remains to be determined what this second activity is and whether it requires both RRM1 and 2, the short linker peptide between these RRM1 or all of these sequences. Such studies will likely require detailed biochemical analysis and thus are well beyond the scope of this initial characterization; however, we predict that future study of

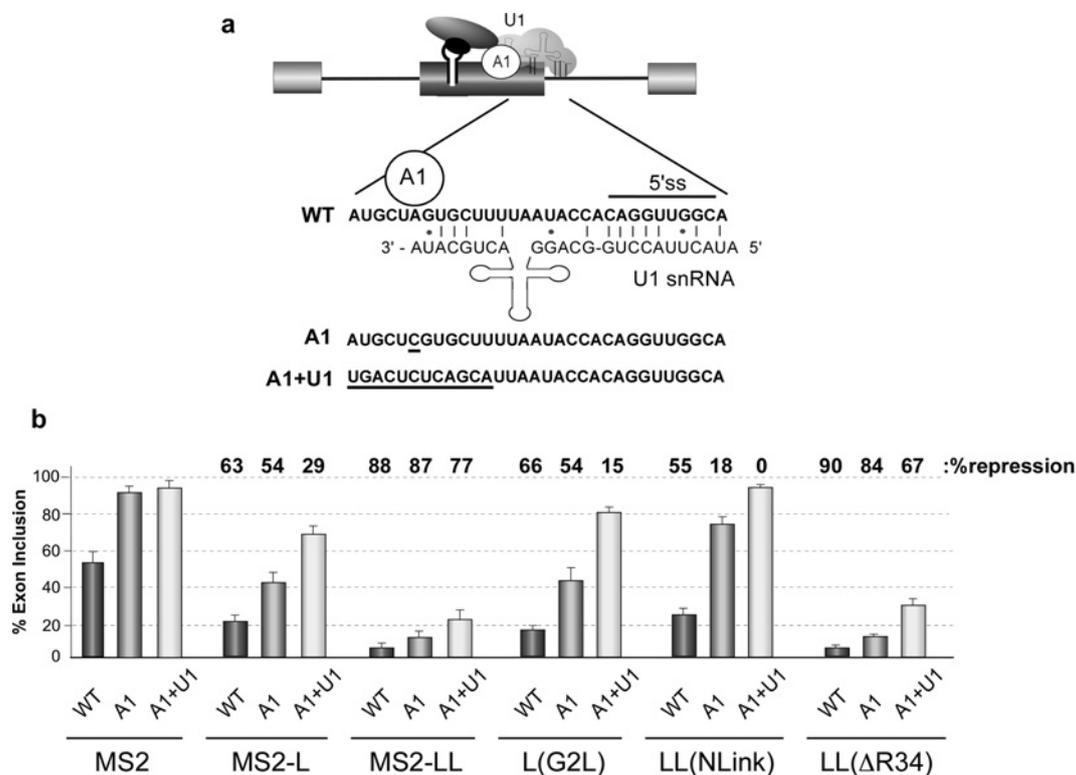


Figure 6 Repressive activity of hnRNP LL is distinct from mechanism of hnRNP L function

(a) Illustration of mechanism described previously for hnRNP L-mediated exon repression in which hnRNP L (dark grey oval) bound upstream in exon recruits hnRNP A1 (white circle) to a UAGUG element at the 3'-end of the exon. hnRNP L and A1 then together induce an altered conformation of the U1 snRNA bound to the 5' splice site (5'ss), resulting in aberrant contacts between the U1 snRNA and the exon which preclude appropriate release of the U1 snRNP in spliceosome assembly. Mutations that disrupt binding of hnRNP A1 (A1) or disrupt both hnRNP A1 binding and the extended base-pairing of U1 (A1 + U1) are shown. (b) Histogram of the mean percentage inclusion (\pm S.E.M.) of MS2E4 from at least three independent replicates of RT-PCR as in Figure 3(B), using mutant versions of the central MS2E4 exon shown in (a) and proteins described in previous Figures. The percentage repression of each mutant construct by each fusion protein is shown above histogram and was calculated as $[(\%inclusion)_{MS2} - (\%inclusion)_{MS2-fusion}] / [(\%inclusion)_{MS2}]$. WT, wild-type.

the RRM 1 and 2 region of hnRNP LL will unveil new and exciting insights into the mechanisms of splicing regulation.

AUTHOR CONTRIBUTION

Ganesh Shankarling carried out all experiments, and Kristen Lynch and Ganesh Shankarling designed the project, analysed the data and wrote the paper.

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