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Tissue-specific Splice Variants of HARE/Stabilin-2 are Expressed in Bone Marrow, Lymph Node, and Spleen

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Abstract

The hyaluronan receptor for endocytosis (HARE), or Stabilin-2, is the mammalian endocytic clearance receptor for HA, heparin, advanced glycation end-products, acetylated and oxidized low-density lipoproteins and collagen N-terminal propeptides. This large 2551 amino acid receptor is encoded by a gene that covers over 180 kbp on human chromosome 12 and is predicted to be composed of 69 exons. Due to the expression profile of this gene and the number of exons it contains, we hypothesized that splice variants of *stab2* are encoded in these tissues. In addition, a correlation between alternative splice variants and cancer progression has been shown in other HA receptors such as RHAMM and CD42. In this study, two methods were utilized in identifying and/or isolating the HARE splice variants. The first method used primer sets to amplify the 190-HARE encoding region that could contain splice junctions; therefore, they could be removed from the gel, purified, and sequenced. Five splice variants were detected in that manner. In the second approach, the entire open reading frame of HARE was amplified. This allowed four splice variants with extensive exon splicing to be isolated. After the splice variants were sequenced, three were cloned into a mammalian expression vector. Next, stable cell lines expressing the variants were created in order to determine stable protein expression. In this study, the splice variants were found to be tissue specific in most cases. This means that tissue specific regulatory splicing mechanisms may lead to differences in functionality between the splice variants.

Keywords

hyaluronan; receptor; HARE; splice variants; Stabilin-2

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INTRODUCTION

Since the initial purification, identification and molecular cloning of the rat [1, 2, 3] and human HARE [4, 5], which is also designated Stabilin-2 [6, 7, 8] or FEEL-2 [9], much has been learned about its ligand binding activities and multiple functions. Although first identified as the mammalian endocytic clearance receptor for HA and chondroitin sulfates by Laurent and co-workers [10, 11, 12, 13, 14], HARE/Stab2 is now known to bind >14 distinct ligands, to be a signaling receptor and is involved in a growing list of diverse physiologic functions. Human HARE/Stab2 is encoded by a 180 kbp gene on chromosome 12q23 consisting of 69 exons, and the full-length 7653 bp mRNA is translated into a 2551 aa 315-kDa Type I glycoprotein receptor, and trafficked to the plasma membrane and intracellular endosomal compartments. In stably transfected Flp-In 293 cells, 315-kDa HARE (315-HARE) cDNA is expressed mostly as the full-length polypeptide with a minor fraction that is post-translationally cleaved to produce a 190-kDa HARE (190-HARE) type I receptor [15] that is functional in the absence of the 315-HARE [5].

The 315-HARE and 190-HARE isoforms both bind to HA with high affinity, K_d values of 7–42 nM [15], and mediate very rapid coated pit targeted endocytosis of HA and other ligands including chondroitin sulfates A, C, D and E, dermatan sulfate, acetylated & oxidized low density lipoprotein, advanced glycation end-products, and heparin [6, 7, 9, 16, 17, 18, 19, 20]. Both HARE isoforms show HA-dependent intracellular activation of ERK1/2 [21], which requires the HA-binding Link domain of HARE in its properly glycosylated state [22]. HARE/Stab2 also binds and mediates phagocytosis of bacteria [9] and apoptotic cells displaying cell surface phosphatidylserine [23, 24], and mediates lymphocyte adhesion to sinusoidal endothelium by interacting with $\alpha\text{M}\beta\text{2}$ [25] and $\alpha\text{5}\beta\text{5}$ integrins [26].

Over the past 10–15 years, the roles of HA in cancer biology and the importance of tumor-associated alternative splice variants have gained considerable attention. The most studied HA receptor showing tumor-associated increased transcript diversity for multiple splice variants is CD44 [27] (e.g. CD44v6 mRNA is increased 82% in colon cancer [28] and CD44v10 strongly correlates to bladder epithelial carcinoma recurrence [29]). In addition, up-regulation of RHAMM (Receptor for HA Mediated Mobility) and its variants correlate to cancer progression [30, 31, 32]. Over-expression of a RHAMMv4 variant, which is primarily found in the cytoplasm in contrast to the native receptor in the plasma membrane, promotes cell migration and proliferation via Ras and ERK signaling [33].

Here we report the first identification of nine different tissue-specific splice variants of human HARE found in cDNA pools of healthy spleen (six), lymph node (three), and bone marrow (one). Three of these splice variants have been cloned into mammalian-based vectors for expression in human cell culture.

MATERIALS AND METHODS

Materials and Solutions/Buffers

Flp-In 293 cells, serum, media, Hygromycin B, Zeocin, glutamine, plasmid expression vectors, and supercompetent TOP10 *E. coli*, and Lipofectamine2000 were from Invitrogen/Gibco (Carlsbad, CA). Polyclonal goat anti-V5 affinity purified antibody and goat anti-V5 antibody resin were obtained from Bethyl Labs (Montgomery, TX). All chemical reagents were purchased for Sigma unless otherwise noted. Buffer compositions may be found in previous publications [5, 15].

Amplification of variant HARE cDNA species

Five pairs of primers were used in separate reactions to amplify adjacent ~1 kb regions of the 190-HARE cDNA (Table I). The cDNA pools used as the source of variant HARE cDNAs were Marathon-Ready cDNAs (Clontech, now part of Takara Corp.) from human spleen (Cat. #639312), human lymph node (Cat. #639332), and human bone marrow (Cat. #639316). In order to detect rarer cDNAs that might require a second round of PCR amplification, the region immediately below the wildtype band was cut from the gel using a clean razor blade and nucleic acids were purified from the agarose gel slice using a GeneClean Turbo kit and subjected to a second round of PCR, with conditions identical to the first PCR round. Visible bands, stained with ethidium bromide, were excised and purified with a GeneClean Turbo kit (MPBio) and directly sequenced using the forward PCR reaction primer. PCR products from reactions containing the ORF flanking primers that were HARE related and ready for protein expression were immediately cloned into pcDNA5/FRT/V5-6xHis-TOPO for recombinant protein expression studies.

Transient Transfections

293 Flp-In cells were grown to 50% confluency in DMEM/8% FBS supplement with 100 ug/ml Zeocin in 24 well plates 1 day prior to transfection. For each representative well to be transfected, 1 µg plasmid DNA was mixed in 50 µl 150 mM NaCl solution pH 7.3 followed by the addition of 3.3 µl of ExGen500 (Fermentas) and incubated in a 1.5 ml tube for 15 minutes. The entire solution was added to the cells and the plate was centrifuged for 5 minutes at 250×g. Expression of recombinant protein typically took 48 hours.

Selection and Verification of 35/66 and 13/69 stable cell lines

Cell lines were produced and characterized as described by Harris *et al.* [5]

Western Blot Assays

Cell lysates, immunoprecipitates using the anti-V5 antibody, or eluate from the nickel affinity chromatography were mixed with 4x Laemmli sample buffer without reducing agent and analyzed by a 5% or 10% SDS-PAGE gel.

RESULTS

The full-length *hare* mRNA contains 69 exons, which are remarkably similar in size (46 to 197 bp) as predicted by the NCBI database (Accession #NM_017564, supplemental Figure

1). We employed two strategies to amplify low-copy spliced cDNAs from Marathon human cDNA pools from both spleen and lymph node. For our first strategy, we used 5 primer sets (Table 1) encompassing the 190-HARE encoding region to amplify regions of <1100 bp that may have only one exon excision (Figure 1a). This was performed to have a control for each primer set in the amplification reaction as well as amplifying a region small enough so that a transcript with an exon deletion could be detected by separation on agarose gel electrophoresis. The control reaction contained only the recombinant 190-HARE cDNA so that the abundant wildtype cDNA in the Marathon pools could be identified and separated from any splice variants. All of the reactions were separated by 1% gel electrophoresis (Figure 1b). Since any potential splice variants would be smaller than wildtype, we excised the region below the wildtype band with a razor blade, purified all nucleic acids from the gel, and repeated the amplification reaction. The second reaction produced one to several bands in 3 of the 4 lanes that were not seen from the first reaction (Figure 1c). Each DNA band was purified from the gel and sequenced. Some bands were the result of non-*STAB2* gene specific primer annealing to cDNAs that encoded enzymes in other metabolic pathways. However, the brightest of the bands in the 2nd amplification reactions usually were splice variants for HARE (Figure 1c). Some of the splice variants were of sufficiently high copy number to detect from the initial amplification reaction (Figure 1d, gray arrow) and distinguished from the wildtype copy (Figure 1d, black arrow).

Our second strategy was to use a primer set that flanked and amplified the entire open reading frame for *STAB2* in spleen, lymph node, and bone marrow cDNA pools. This method was used to identify and isolate four splice variants with extensive exon splicing in addition to obtaining a cDNA that could be expressed in 293 Flp-In cell lines. In most cases, the splice variants were tissue specific. Using the same amplification reactions outlined above in spleen, lymph node, and bone marrow cDNA pools, we identified the presence of one of the variants in either spleen (Figure 1d) or in lymph node, but not in both. This was true for all but one of the variants (35/66, found in LN and BM) leading us to believe that there are different tissue specific regulatory splicing mechanisms employed and that the splice variants may be functionally unique (Figure 1e). Table 2 gives more details for each splice variant discovered to date.

To name the splice variants, we used the backslash (/) to indicate the region that was spliced out of the original transcript. For example, variant 1/63 is missing exons 2–62 and exons 1 and 63 are fused in frame. A schematic representation of all the splice variants is illustrated in Figure 2. The first four variants contain the entire open reading frame and three of these were expressed in mammalian cells. The remaining five variants consist of regions that were identified by the flanking primers outlined in the first amplification strategy and are partial sequences of the variant. The yellow boxes represent frame shifts due to the exon fusion in the splicing region of the DNA that resulted in a premature stop codon. The arrows indicate the areas in which the remaining splice variant cDNA would presumably exist, but remains unidentified due to the limitations imposed by the first strategy.

Splice variants are stably expressed in cell culture

After the sequence was confirmed, the cDNAs for variants 1/63, 13/69, and 35/66 were cloned into the mammalian expression vector, pcDNA5/FRT/V5-6xHis-TOPO which is suitable for both transient and stable transfections. This procedure was performed to ensure that the protein was folded and expressed in the correct manner as previous attempts to clone deletion mutants of HARE were quickly degraded, probably as a result of misfolding, and not expressed in cell lines. Three splice variants were expressed in transient transfections and detected with the anti-V5 antibody. Stable cell lines expressing these variants were created to determine stable protein expression. In Figure 3a, variant 1/63 (gray arrow) is membrane bound in contrast to variant 13/69 (black arrow) which is secreted from the cells. Both variants were immunoprecipitated with the anti-V5 antibody resin, separated by 10% SDS-PAGE, and detected with anti-V5 antibody. In Figure 3b, variant 35/66 is also membrane-bound and cell lysates from wildtype (black arrow) and 35/66 stable clone 8 (gray arrow) were separated by 5% SDS-PAGE and detected by anti-V5 antibody.

Three of the eight monoclonals raised against the rat 175-kDa HARE protein cross-react with both isoforms of wildtype human HARE [5, 15]. Cell lysates from stably expressing 35/66 cell lines were tested against mAbs 30, 154, and 159 (Fig. 4a). Only mAb159 reacted against multiple clones of variant 35/66 (Figure 4b) as compared against the control antibody, anti-V5 (Figure 4c). Variants 1/63 and 13/69 did not react with any of the mAbs (not shown). This data, in conjunction with other unpublished data from our laboratory, suggests that antibody 159 recognizes an epitope, which is very close to the transmembrane region and most likely located within exon 66.

DISCUSSION

Regulation of post-translational processes in different tissues may affect how HARE binds to HA and other GAGs. This may also apply to post-transcriptional modification of the HARE mRNA where numerous splice variants may be expressed differentially in normal tissues or altered in cancerous cells. Although previous experiments using anti-HARE mAb to evaluate HARE-related protein expression were negative for most rat tissues tested, the lack of antibody reactivity for smaller splice variants may be due to lower expression levels of the protein or to missing epitopes in the expressed splice variants [1]. Additional studies confirm that *stab2* mRNA is present in liver, spleen [8] and in bone marrow [34].

The human *stab2* gene annotation in the NCBI database predicts 69 exons in which the sequences of the exon-exon linkages are predicted. The presence of these splice variants in normal tissue used to produce the cDNA pools do not seem to be a result of random ligations or a product of the experimental procedures. The chosen primers merely amplified the region of interest without manipulating the DNA. How do we know that these splice variants are real and not a result of the procedure? First, all but one of the splice variants were spliced at the predicted junctions. The only variant that did not adhere to this parameter was HARE ν (62/67) in which the last 9 codons of exon 62 were lost and most of the codons on the 5' half of exon 67 were also lost to form a hybrid region composing of exons 62 and 67 linked in frame. Theoretically, the resulting protein would be secreted since the transmembrane domain, which is encoded mostly by exon 67, is lost. The second line of

evidence which demonstrates that the formation of HARE splice variants are a real biological process is that HAREv(35/66) has two isoforms on the RNA level encoding the same predicted protein. Version 1 of this variant adheres strictly to the predicted joining of exons 35 and 66 through the pairing of two adenosines. In contrast, version 2 loses the terminal adenosine on exon 35 and gains a terminal guanidine on exon 65 resulting in the pairing of two guanidines and preserving the reading frame (Supplemental Figure 2). Both splicing versions of HAREv(35/66) produce a translated product with version 1 used in the experimental procedures of this paper.

The full-length HARE protein contains 204 cysteine amino acid of which over half are known to fold in 21 EGF/EGF-like domains organized in 4 clusters and one Link domain near the transmembrane domain. The remaining 74 cysteine residues are thought to be participating in either disulfide bonds with each other or modified in various forms of cysteine oxidation due to the oxidizing conditions found in the ER [35]. The best engineered *E. coli* strains designed specifically for protein folding do not match the fidelity of the mammalian ER calnexin/calreticulin system for HARE expression [25, 36]. Thus, an improperly folded HARE protein does not progress to the Golgi and is degraded through the ERAD system [37]. Since the overall physical structure and cysteine bonding pattern is not established for HARE or for Stabilin-1, the closest orthologue of HARE, the construction of deletion mutants using the exon junctions have proven quite advantageous when evaluating specific domains.

The majority of the protein comprising v35/66 (exons 1–35/66–69), is absent from the 190-HARE isoform (exons 32–69). We had previously tested both 190- and 315-HARE isoforms for a number of ligands to determine binding sites. Hyaluronan, CS-A, CS-C, and CS-D all bind within the Link domain (green region, Fig. 2) and heparin and acetylated LDL strictly bind to a region upstream of the Link domain [16]. Since the heparin binding site(s) have not been elucidated, we set out to determine if v35/66 may contain one or more of these sites. Through numerous binding and endocytosis assays, it was determined that v35/66 does not bind heparin; therefore, the heparin-binding site(s) are situated all within the 190-HARE protein (data not shown). This further confirms our previous data which demonstrated that both HARE/Stab2 isoforms have approximately the same binding constants and capacity for heparin ligands [17].

Further studies are needed to define the physiological function of these splice variants and to screen malignant tissues to determine if there is a differential expression compared to healthy tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HA	Hyaluronic Acid
HARE	Hyaluronic Acid Receptor for Endocytosis
190-HARE	the small isoform of HARE
315-HARE	the full-length isoform of HARE
hnRNA	heteronuclear RNA
mAb	monoclonal antibody
NCBI	National Center for Biotechnology Information

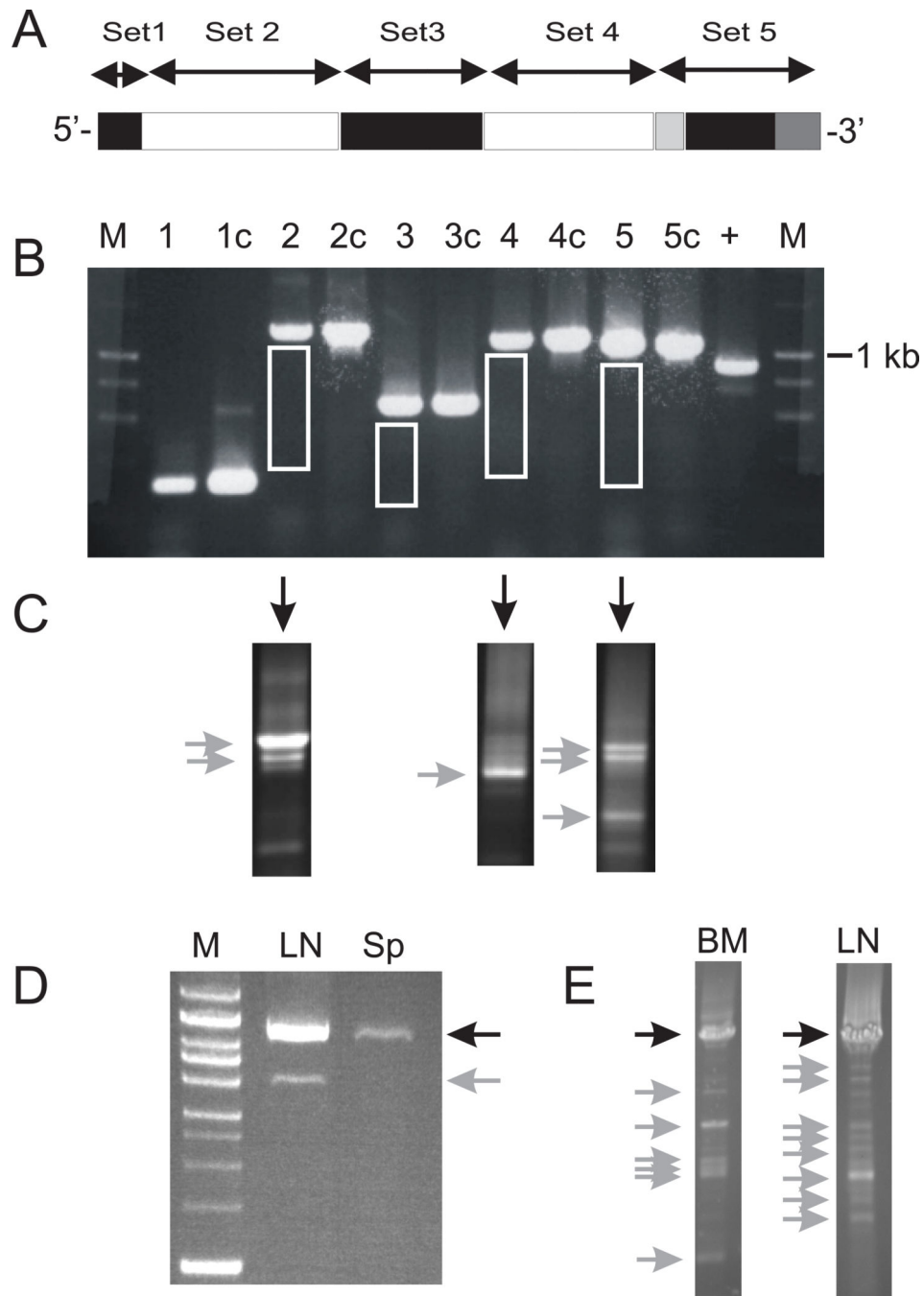
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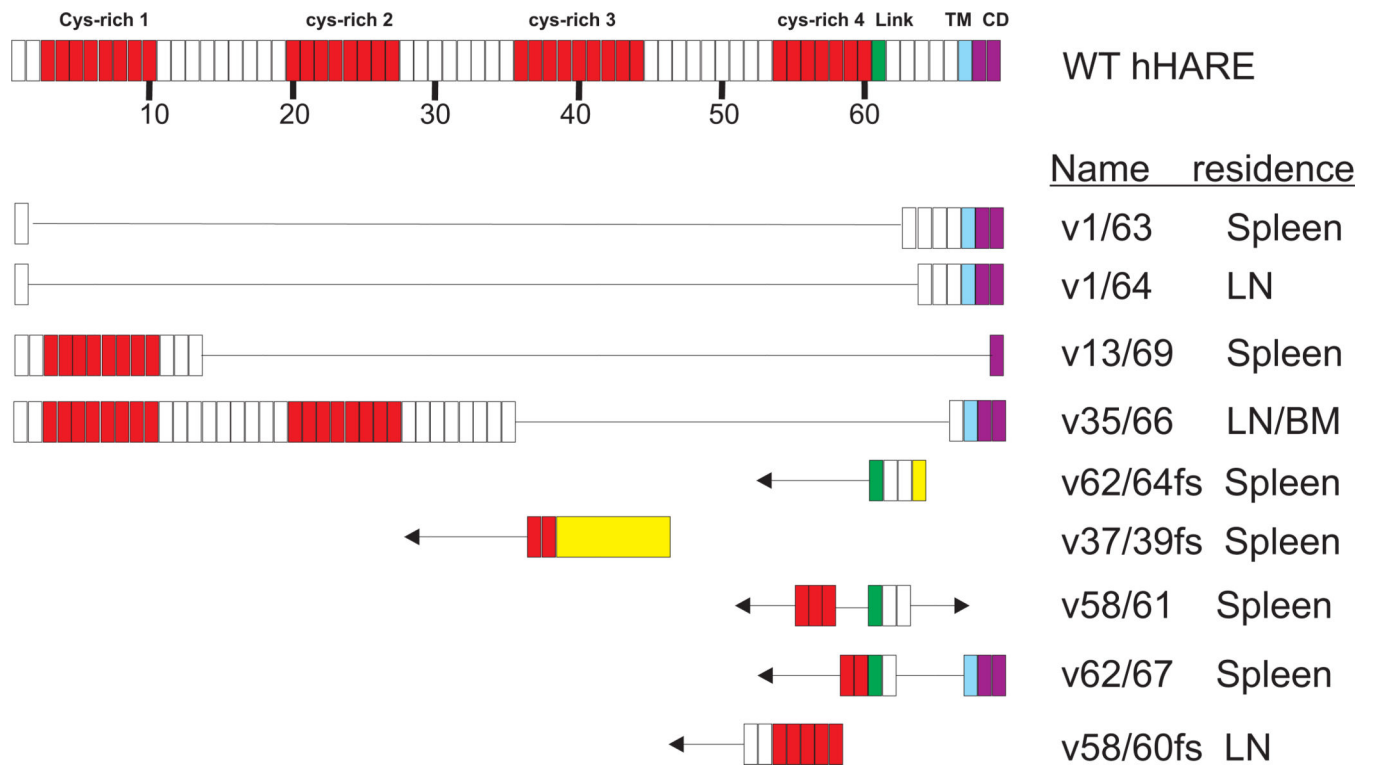
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- HARE has 69 exons.
- Nine splice variants were detected by PCR.
- HARE undergoes specific splicing.

**FIGURE 1.**

Strategy for identifying and cloning splice variants from human cDNA pools. A) Regions of the 190-HARE targeted for amplification. B) The agarose gel indicating both control (lanes marked with "c") and samples. The "+" is the positive GAPDH control for the PCR conditions with the cDNA pools. The white boxes indicate areas of the gel that were cut out with a standard razor blade and purified for nucleic acids. C) Re-amplification results from areas cut from the gel as indicated in part B. D) Flanking primers for HARE in both lymph node and spleen cDNA pools. The black arrow indicates the WT cDNA product and the

gray arrow indicates the splice variant. E) Side-by-side comparison of the PCR results on agarose gel of the bone marrow and lymph node cDNA pools using HARE flanking primers. Black arrow = WT, gray arrows = potential splice variants.

**FIGURE 2.**

An illustration of the HARE splice variants identified to date. Each rectangle represents an exon. Red = EGF/EGF-like clusters, white = Fasciclin domains, green = Link domain, blue = transmembrane domain, purple = cytoplasmic domain, yellow = newly translated portion of a variant caused by a frameshift at the splice junction.

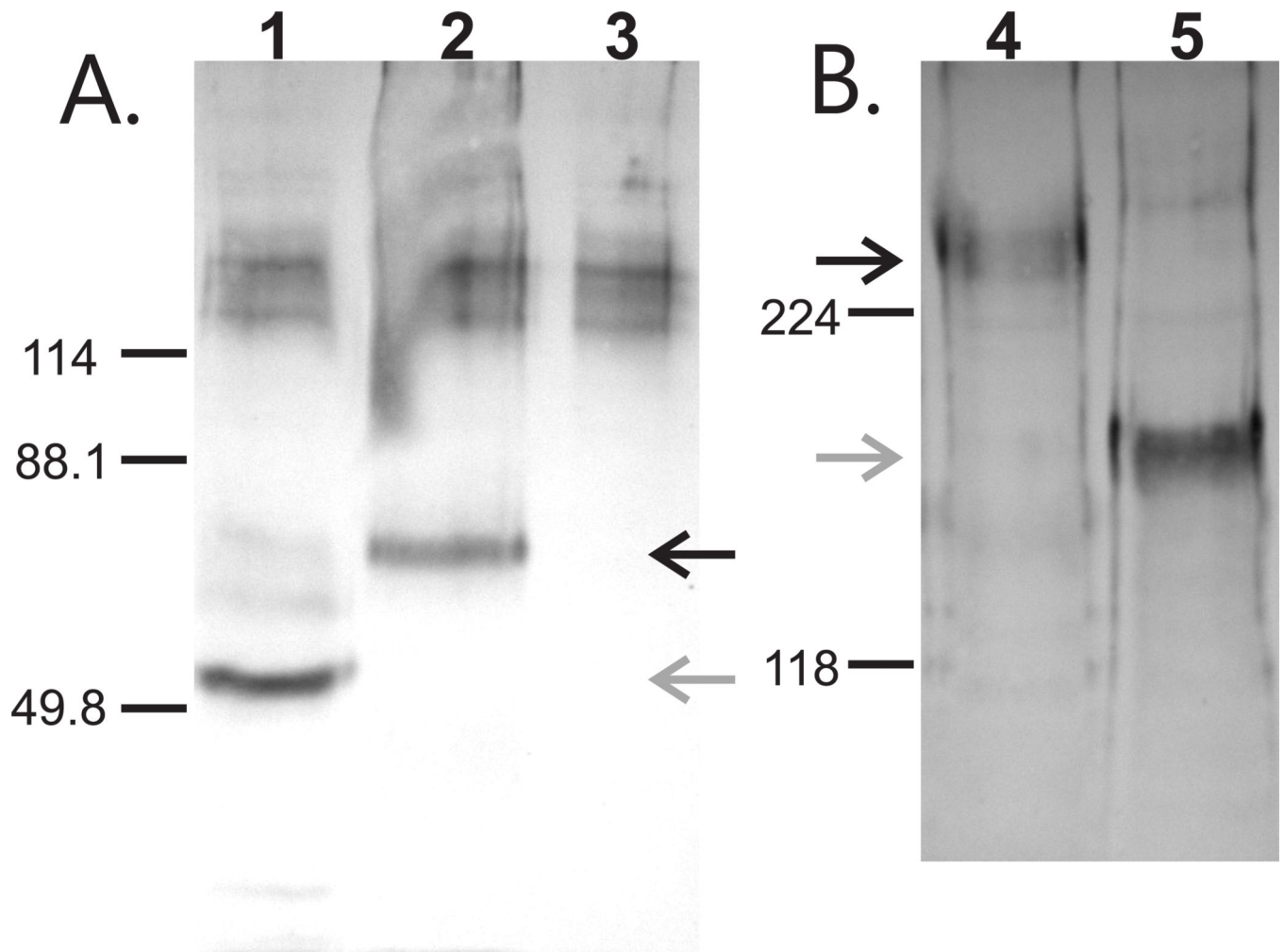


FIGURE 3.

Expression of splice variants in stable cell lines. A) Immunoprecipitation followed by 10% SDS-PAGE, blotting, and probing with anti-V5 of HAREv1/63 (lane 1) and HAREv13/69 (lane 2) in addition to anti-V5 resin alone (lane 3). Arrows indicate the respective recombinant proteins. B) Cell lysates from WT (lane 1) and HAREv35/66 (lane 2) are separated by 5% SDS-PAGE, blotted and probed with anti-V5.

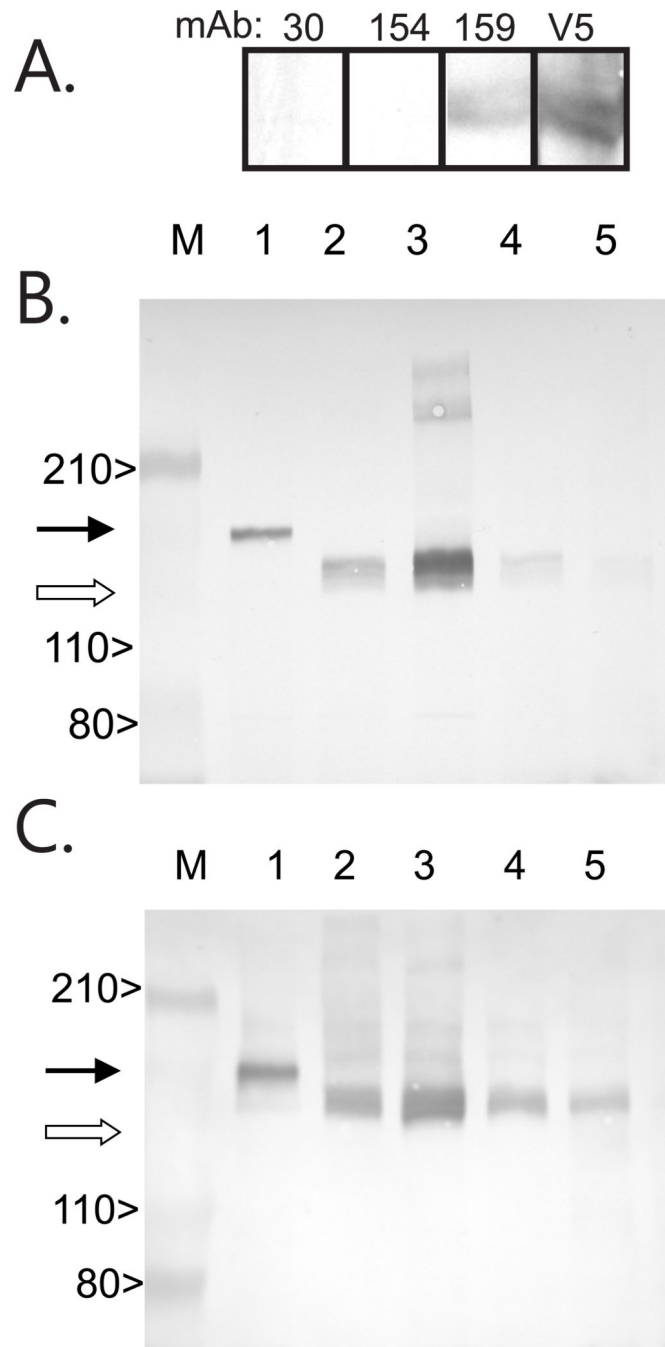


FIGURE 4. HAREv35/66 reacts with anti-rat HARE mAb159. A) The three monoclonal antibodies (30, 154, 159) that react with human HARE were also reacted against HAREv35/66 cell lysate. Cell lysates from the following cell lines (lane 1=190-HARE, lane 2 = HAREv35/66 clone 5, lane 3 = clone 8, lane 4 = clone 13, lane 5 = clone 18) were probed with B) mAb159 and C) anti-V5.

Table I
190-HARE Primers used to amplify variants

The five overlapping sets (numbered 1–5) of forward (F) and reverse (R) primers spanning the complete 190-HARE ORF cDNA sequence (amplified using the last primer pair) are indicated in the schematic in Fig. 1. The numbering for the starting 5' nucleotide in each primer is based on the full-length 315-HARE coding sequence.

Primer Set	T _m (°C)	Primer start nucleotide number	Primer Sequence
1	65	F:3406 R:3711	F: 5'-TTCGAATCCTTACCAAACCTGCTCATGCG R: 5'-GTCATTATGGAGAAAGAAGCTCAGGAAATAGGAGAAACC
2	65	F:3673 R:4890	F: 5'-GGTTTCCTATTTCCTGAGCTTCTTTCTCCATAATGAC R: 5'-GAAGGGGCCCGGTCCGACCAGATCTTTCAC
3	70	F:4861 R:5520	F: 5'-GTGAAAGATCTGGTCGGACCGGGCCCTTC R: 5'-CAGCTCTGAACCCTGCAGGGTCTCCAGGC
4	65	F:5491 R:6621	F: 5'-GCCTGGAAGACCCTGCAGGGTTCAGAGCTG R: 5'-GCCAGTGGCGATCGTAGATGGAACAC
5	65	F:6595 R:7653	F: 5'-GTGTTCCATCTACGATCGCCACTGGGC R: 5'-CGGGATCCCAGTGTCTCAAGGGGTCATTG
315-HARE ORF	62	F:1 R:7653	F: 5'-CGGGATCCATGATGCTACAACATTTAGTAATTTTTGTCTTGG R: 5'-CGGGATCCCAGTGTCTCAAGGGGTCATTG

Table 2

Splice variants identified from human spleen and lymph node cDNA pools.

Name of Variant	Exon(s) spliced out	Frameshift	Inclusion of full reading ORF	Resident Organ	Detected in first round of PCR
hHAREx(62/64)fs	63	Yes	No	Spleen	Yes
hHAREx(37/39)fs	38	Yes	No	Spleen	No
hHAREx(58/61)	59,60	No	No	Spleen	No
hHAREx(-62/67)*	63-66	No	No	Spleen	No
hHAREx(1/64)	2-63	No	Yes	Spleen	Yes
hHAREx(13/69)	14-68	No	Yes	Spleen	Yes
HHAREx(1/63)	2-62	No	Yes	LN	Yes
hHAREx(35/66)	36-65	No	Yes	LN/BM	Yes
hHAREx(58/60)fs	59	Yes	No	LN	No

* splice variant that does not follow splicing rules.