A Glycine-Rich Domain of hnRNP H/F Promotes Nucleocytoplasmic Shuttling and Nuclear Import through an Interaction with Transportin 1^{∇†}

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Heterogeneous nuclear ribonucleoprotein (hnRNP) H and F are members of a closely related subfamily of hnRNP proteins that are implicated in many aspects of RNA processing. hnRNP H and F are alternative splicing factors for numerous U2- and U12-dependent introns. The proteins have three RNA binding domains and two glycine-rich domains and localize to both the nucleus and cytoplasm, but little is known about which domains govern subcellular localization or splicing activity. We show here that the central glycine-tyrosine-arginine-rich (GYR) domain is responsible for nuclear localization, and a nonclassical nuclear localization signal (NLS) was mapped to a short, highly conserved sequence whose activity was compromised by point mutations. Glutathione *S*-transferase (GST) pulldown assays demonstrated that the hnRNP H NLS interacts with the import receptor transportin 1. Finally, we show that hnRNP H/F are transcription-dependent shuttling proteins. Collectively, the results suggest that hnRNP H and F are GYR domain-dependent shuttling proteins whose posttranslational modifications may alter nuclear localization and hence function.

Most genes in metazoans contain multiple introns that need to be removed by mRNA splicing to generate mature mRNA. Alternative splicing, the process whereby multiple mRNAs (and thus proteins) are produced from the same gene through inclusion/exclusion of various exons (7), is widespread, and it has become clear that alternative splicing plays a large role in regulated gene expression and accounts for enormous protein diversity (9, 38, 62). The heterogeneous nuclear ribonucleoprotein (hnRNP) family of factors represents an important group of proteins that bind splicing regulatory elements to positively and negatively influence alternative splicing; hnRNP A1 and hnRNP H/F are particularly important in this regard (16–18, 42, 48, 50, 51). Binding to exonic splicing silencers and oligomerization of hnRNP A1 along the exon are thought to sterically displace positively acting factors, such as serine-arginine (SR) proteins (61, 82), or alternatively, hnRNP A1 may operate to repress splicing by binding to intronic splicing silencers, which may loop out the exon (8, 60). This looping mechanism might also account for a positive role for hnRNP A1 and H/F in splicing control through interactions with sites at each end of long introns, thus bringing splice sites together (24, 47).

In addition to their influence on splicing through recognition of specific binding sites within target RNAs, subcellular localization of hnRNP proteins has emerged as a mechanism to regulate splicing. While essentially nuclear at steady state, sev-

eral hnRNP proteins (hnRNP A1/A2, K, D, E, and I) continuously shuttle between the nucleus and cytoplasm (55). hnRNP A1 is particularly well studied in this regard and shuttles through the action of a bidirectional transport peptide called M9 (65). While the export activity of M9 is not well understood, nuclear import of hnRNP A1 is mediated by the interaction of the M9 peptide with the import receptors transportin 1 and 2 (Trn1/2) (26, 31, 67, 68). Import can be regulated, for example, in response to osmotic stress where activation of the p38 MKK_{3/6}/p38 signaling pathway results in hyperphosphorylation of hnRNP A1 in a region near the M9 sequence, which disrupts the interaction with transportin and results in cytoplasmic accumulation (1, 29, 77). Significantly, this redistribution altered alternative splicing of a test substrate, suggesting that more global changes in cellular splicing might occur because of changes in localization.

The related members of the hnRNP H family (hnRNP H, H', F, and 2H9) are evolutionarily conserved in vertebrates and influence mRNA capping, splicing, polyadenylation, export, and translation. Positive and negative influences of these proteins on alternative splicing through binding to G-rich sequence elements have been widely reported (10, 11, 14–17, 27, 32, 46, 48, 50, 53, 56, 58, 79), and recent global analysis of splicing in cells in which hnRNP H was knocked down by RNA interference (RNAi) indicates that a large number of alternative splicing events are influenced by hnRNP H (81). In several cases, it appears that hnRNP H and F are partially interchangeable for regulation of U2-dependent introns (17, 27, 32, 50, 69, 79, 81), whereas only hnRNP H, and not F, could promote U12-dependent splicing (53). hnRNP H/F are also nuclear at steady state in HeLa cells, but both proteins are localized to a degree in the cytoplasm in certain tissues, and substantial cytoplasmic relocation (especially for hnRNP F) is reported in some tumors (4, 34, 36, 40, 49). These localiza-

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tion differences could have a profound impact on the global splicing profile in healthy and diseased tissues (12).

The hnRNP H'/H/F proteins have three highly homologous quasi-RRM-type RNA binding domains (RRM stands for RNA recognition motif) that account for specific binding to G-rich RNA (20, 35), and two additional poorly characterized glycine-rich domains (GRDs) that are referred to as the GYR and GY domains, reflective of their amino acid content. Presumably, these GRDs are involved in protein-protein interactions and account for the localization characteristics of hnRNP H/F, and differences in these domains might underlie the differential effects of hnRNP H and F on U12-dependent splicing (53). In this report, we provide evidence that the GYR domains of hnRNP H and F harbor a phylogenetically conserved nuclear localization sequence (NLS) that interacts with the import receptor Trn1. It was also established that hnRNP H and F are shuttling proteins and that shuttling is mediated by the GYR domain. Posttranslational modifications have the potential to impact the transport activities ascribed to the GYR domain and thus influence alternative splicing.

MATERIALS AND METHODS

Plasmids. pcDNA-H, pcDNA-MS2-H, and pcDNA-MS2-F have been described previously (52, 53). pGST-Trn1 was a kind gift from J. Steitz (Yale University, New Haven, CT), pcDNA-hnRNP C and pcDNA-hnRNP A1 were kind gifts from G. Dreyfuss (University of Pennsylvania, Philadelphia, PA). pcDNA-MS2-HΔGY (pLY86) was made by replacing the full-length hnRNP H reading frame in pcDNA-MS2-H with an XhoI-BamHI PCR fragment encoding amino acids (aa) 1 to 367 (all primer sequences available upon request). pMS2H-ΔR3GY (aa 1 to 292; pLY87), pMS2H-RRM1-2 (aa 1 to 202; pLY84), pMS2H-GYR-R3-GY (aa 194 to 449; pLY88), pMS2H-R3-GY (aa 284 to 449; pLY89), pMS2H-GY (aa 367 to 449; pLY90), and pcDNA-MS2-Hgyr (aa 194 to 292; pLY99) were made from XhoI-BamHI PCR fragments encoding the indicated amino acids, which were used to replace the full-length hnRNP H reading frame in pcDNA-MS2-H. pMS2-GFP (pLY85) was made by replacing the XhoI-BamHI(blunt) fragment in pcDNA-MS2-H with an XhoI-NotI(blunt) fragment from pEGFP. pGFP-H (pLY119), pGFP-HRRM1-2 (pLY96), pGFP-HGYR (pLY98), and pGFP-HGY (pLY97) were made by moving XhoI-BamHI fragments from the above pcDNA constructs into pEGFP-C3. pGFP-F (aa 1 to 416; pLY120), pGFP-FRRM1-2 (aa 1 to 202; pSF6), pGFP-FGYR (aa 194 to 292; pSF5), and pGFP-FGY (aa 367 to 415; pSF7) were made by moving XhoI-BamHI PCR fragments of the indicated regions of hnRNP F into pEGFP-C3. pGFP-HgyrA-F (fragments of the GYR domain, see the amino acids in Fig. 2) were made by inserting XhoI-BamHI PCR products into the same sites of pEGFP-C3. pGFP-HgyrD-P197A, R206A, P209A, Y210A, D211A, R212A, Y246A/E/F, Y276A/E/F were made with XhoI-BamHI PCR fragments of the GYR or GYR-D constructs containing the indicated mutations generated by overlap PCR; fragments were inserted into the same sites of pEGFP-C3. Full-length pGFP-H harboring the Y210A point mutation (pMM368) and deletion of amino acids 205 to 213 (pMM366) were made by overlap PCR. pGST-M9 had an XhoI-BamHI PCR fragment encoding aa 268 to 305 of hnRNP A1 inserted into the same sites of pGEX-6P-1XB, a derivative of pGEX-6P-1 in which the XhoI and BamHI sites were reoriented. Overlapping oligonucleotides containing XhoI-BamHI ends and encoding the simian virus 40 (SV40) NLS (PPKKKKR VEDP) were annealed and inserted into the same sites of pGEX-6P-1XB to generate pGST-NLS. pGST-Hgyr (pCVD1), pGST-HgyrY210A (pCVD11), pGST-HgyrD (pLY162), and pGST-HgyrD-Y210A (pLY165) were made by moving the indicated fragments from the constructs described above into pGEX-6P-1XB.

Recombinant proteins. Proteins were expressed in BL21 cells by induction with 0.1 to 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C, and cell pellets were resuspended in cold 1× phosphate-buffered saline (PBS) supplemented with 1× protease inhibitor cocktail (Calbiochem), 100 mM EDTA, 1.5% *N*-lauryl sarcosine, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated four times for 15 s each time. After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatant was filtered through a 0.45-μm syringe filter and incubated with glutathione-Sepharose 4B beads (GE Healthcare) in PBS for 30 min at 4°C. The beads were eluted three

times in 0.75 ml of elution buffer (50 mM Tris-HCl [pH 8.0], 400 mM NaCl, 20 mM glutathione, and 1 mM dithiothreitol [DTT]). The eluate was then dialyzed against buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, and 10% glycerol overnight at 4°C, then quick-frozen on dry ice, and stored at -80°C.

Transfection, RNA analysis, and immunofluorescence. HeLa cells were seeded at a concentration so that they would reach ~80% confluence the next day and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. For immunofluorescence of myc-tagged proteins, HeLa cells were seeded on coverslips and transfected as described above. The following day, the cells were washed in PBS, fixed in 1% paraformaldehyde in PBS for 30 min, followed by permeabilization using PBS containing 0.5% Triton X-100 for 30 min. Mouse anti-myc monoclonal antibody 4A6 (Upstate Biotech; 1:2,000) in 3% normal goat serum was applied for 60 min at room temperature, and after two washes in PBS, secondary goat anti-mouse antibody labeled with Alexa Fluor 594 (Invitrogen; 1:2,500) was added for 60 min. For endogenous hnRNP H, anti-hnRNP H antibodies (Bethyl Laboratories) were used at 1:2,500, followed by secondary goat anti-rabbit antibodies labeled with Alexa Fluor 594. The cells were washed and stained with 4',6'-diamidino-2-phenylindole (DAPI), and the coverslips were mounted on glass slides. Where indicated, the cells were treated with 5 μg/ml actinomycin D for 4 h and then processed for immunofluorescence.

Protein binding assays. The protein binding assay was done essentially by the method of Rebane et al. (68) except that TEN-100 buffer was used instead of TB buffer and NP-40 was used instead of digitonin. Glutathione *S*-transferase (GST) fusion proteins (5 μg) were bound for 1 h at 4°C to 40 μl of glutathione Sepharose 4B beads (GE Life Sciences) in TEN-100 containing 20 mM Tris (pH 7.4), 0.1 mM EDTA, 100 mM NaCl, and 100 μg/ml bovine serum albumin (BSA) and supplemented with 1 mM PMSF and 1× protease inhibitor cocktail (Calbiochem). The beads were washed twice with 1 ml of TEN-100 and incubated for 2 h at 4°C in 0.4 ml of TEN-100 supplemented with 0.5% NP-40, 1 mM PMSF, and 1× protease inhibitor cocktail (Calbiochem) plus 50 μl HeLa cell nuclear extract prepared by the method of Dignam et al. (19). The beads were then washed four times with 0.5 ml of TEN-100 plus 0.5% NP-40 and boiled in 40 μl of 1× SDS solubilizing buffer. The samples were then analyzed by 12% SDS-PAGE followed by Western blotting with a mouse anti-Trn1 IgG (Sigma; 1:2,500) and a secondary goat anti-mouse IgG labeled with horseradish peroxidase (HRP) (Sigma; 1:10,000). The blot was developed using SuperSignal West Pico chemiluminescent substrate (Pierce).

Heterokaryon shuttling assay. HeLa cells were seeded in antibiotic-free minimal essential medium (MEM) supplemented with 10% fetal bovine serum. The cells were seeded at a concentration so that they would reach 80 to 90% confluence the next day. The cells were then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications and allowed to grow overnight. The transfection efficiency was checked the next day by examining the expression of the green fluorescent protein (GFP) control. The cells were then grown on coverslips overnight and incubated for several hours with an equal number of mouse NIH 3T3 cells, followed by incubation for 30 min in medium containing 100 μg/ml cycloheximide. The cells were rinsed with PBS, fused in PBS containing 50% polyethylene glycol 3350 (PEG 3350) for 2 min, rinsed again with PBS, and incubated in medium containing 100 μg/ml cycloheximide for 60 min. Immunofluorescence was performed as described above by incubating cells with an anti-myc antibody (Upstate; 1:2,000) for 1 h at room temperature, followed by incubation with Alexa Fluor 594-labeled anti-mouse antibody (Invitrogen; 1:2,500) for 1 h at room temperature. The cells were then rinsed twice in PBS, stained with DAPI for 10 min, and mounted on glass slides.

RESULTS

A nonclassical NLS resides within the N-terminal region of the hnRNP H/F GYR domain. hnRNP H and F are primarily nuclear proteins, with variable levels of cytoplasmic localization in various tissues and disease states (34, 36, 40, 49). However, examination of the sequence of hnRNP H/F did not reveal a classical Lys-rich NLS. To map an NLS, several myc-tagged MS2-hnRNP H fusion proteins (Fig. 1A) were transfected into HeLa cells and localized by immunofluorescence microscopy for the myc epitope; nuclei were marked by DAPI staining. MS2-GFP was distributed throughout the cell, and as expected, hnRNP H and F were nuclear (Fig. 1B), and the

FIG. 1. The GYR domain of hnRNP H is necessary and sufficient for nuclear localization. (A) Schematic representation of the myc-tagged hnRNP H proteins. The domains and amino acid coordinates are indicated. (B) The indicated myc-tagged MS2 fusion proteins (or MS2-GFP) were expressed in HeLa cells, and protein localization (protein panels) was determined by immunofluorescence microscopy with an antibody to the myc epitope (or direct fluorescence for GFP). Cells were also stained with DAPI as indicated to mark the nuclei (arrows in each panel). In addition to full-length hnRNP H, the additional constructs contain various domains: C-terminal deletions that lacked the GY domain (Δ GY), RRM3 and GY (Δ R3-GY), or contained just RRM1 and 2 (R1-2), and N-terminal deletions that lacked RRM1 and 2 (GYR-R3-GY), RRM1 and 2 and the GYR domain (R3-GY), or fusions of just the GY and GYR domains. qRRM1, quasi-RRM1. (C) Full-length hnRNP H or RRM1 and 2, the GYR domain, or the GY domain, were expressed as GFP fusion proteins in HeLa cells, the proteins were visualized by fluorescence microscopy (protein panels), and nuclei were marked by DAPI stain (arrows in each panel).

FIG. 2. Localization of an NLS within the GYR domain of hnRNP H. (Top) Schematic of full-length hnRNP H with amino acid coordinates, and an enlargement depicting the GYR domain regions (A to F, amino acids indicated), which were fused to GFP. The subcellular localization of the constructs is summarized to the right of the schematic as follows: N, nuclear; C, cytoplasmic. (Bottom) The indicated portions of the GYR domain (aa 194 to 292; A to F) were expressed as GFP fusions in HeLa cells and localized by fluorescence microscopy. The nuclei are indicated by arrows based on DAPI staining of the same samples.

presence of the N-terminal MS2 moiety had no effect on localization (see Fig. S1 in the supplemental material). Examination of numerous deletion constructs showed that only proteins containing the GYR domain were nuclear and that the GYR domain alone was sufficient for nuclear localization. Proteins smaller than ~50 kDa can diffuse into the nucleus, but the nuclear localization of GYR was not merely a consequence of its small size (~28.8 kDa), since the smaller GY protein (~26.2 kDa) was distributed throughout the cell. The RRM1-2, GYR, and GY domains of hnRNP H (and hnRNP F [see Fig. S2 in the supplemental material]) were also fused to

GFP, and again, the GYR domain was necessary and sufficient to direct GFP to the nucleus (Fig. 1C and Fig. S2). These data indicate that a nonclassical NLS resides within the GYR domain of hnRNP H and hnRNP F.

We next localized the NLS within the GYR domain (aa 194 to 292) by fusing a series of GYR domain peptides (denoted A to F) to GFP and imaging fixed cells for GFP; DAPI was used to localize the nuclei (Fig. 2). In contrast to the GFP-GYR peptide located in the nucleus, N-terminal deletion peptides 229-292 (peptide with aa 229 to 292) (A) and 242-292 (B) were distributed throughout the cell. GFP containing C-termi-

FIG. 3. Cross-species amino acid conservation within the GYR domain of hnRNP H and human hnRNP F. At the top is a diagram of hnRNP H with the various domains and amino acid coordinates indicated. Below is a multiple-sequence alignment of the GYR domains of human hnRNP F (top line) and hnRNP H from various species, relative to human hnRNP H. Identical residues are shaded. A highly conserved region in which the indicated residues of human hnRNP H were mutated to alanine is outlined with a thick black line. Mutations outside this region are also indicated. The sequence alignment was produced with ClustalV and the MegAlign program within Lasergene (DNASTAR). Ensembl accession numbers for the sequences are as follows: human hnRNP F (ENSP00000348345), hnRNP H from human (ENSP00000349168), chimpanzee (*Pan troglodytes*) (ENSPTRP00000030096), cow (*Bos taurus*) (ENSBTAP00000012357), dog (*Canis familiaris*) (ENSCAFP00000000471), mouse (*Mus musculus*) (ENSMUSP00000076989), rat (*Rattus norvegicus*) (ENSRNOP00000004688), chicken (*Gallus gallus*) (ENSGALP00000039530), *Xenopus tropicalis* (ENSXETP00000039694), fugu (*Takifugu rubripes*) (ENSTRUP00000002414), and zebrafish (*Danio rerio*) (ENSARP00000027385).

nal GYR deletions (194-272 [C], 194-241 [D], and 194-220 [F]) were largely nuclear, although some cytoplasmic localization was evident with 194-220. The centrally located 229-272 (E) peptide did not confer nuclear localization. These results map a core NLS to aa 194 to 220. Interestingly, this portion of the GYR domain shows the most identity between human hnRNP H and that of more closely related species, and distant orthologs from fugu (39.8%), xenopus (37.5%), and zebrafish (16%), and it has 63% identity to human hnRNP F (Fig. 3). In particular, aa 205 to 213 are invariant in these species with the exception of a glycine-to-serine change at position 208 in zebrafish.

Specific amino acid residues are required for nuclear import. To determine whether amino acids within the highly conserved region (aa 205 to 213) of hnRNP H are required for the nuclear import activity of the GYR domain, several residues were changed to alanine within the context of the 194-241 fragment (D) that showed strong nuclear localization: R206A, P209A, Y210A, D211A, and R212A (Fig. 3). These were expressed as GFP fusion proteins and localized in HeLa cells by fluorescence microscopy. As shown in Fig. 4, compared to the 194-241 peptide, the protein containing D211A was without effect and remained nuclear, whereas the proteins containing

R206A, P209A, Y210A, and R212A showed considerable cytoplasmic signal, but these GFP fusion proteins still showed nuclear accumulation relative to GFP alone. The protein with Y210A appeared to have the most dramatic effect. The effects of these mutations were specific in that mutation P197A, or mutations in conserved residues outside this conserved motif had no effect on nuclear localization (Y246A/E/F and Y276A/E/F [data not shown]). The effects of the mutations on nuclear import were not restricted to the isolated GYR domain, since within the context of full-length hnRNP H, deletion of the conserved domain (Δ 205-213) or incorporation of Y210A resulted in substantial cytoplasmic accumulation. These data demonstrate that an evolutionarily conserved, nonclassical NLS resides within the hnRNP H GYR domain, with a core sequence between amino acids 205 and 213.

The GYR domain of hnRNP H interacts with transportin 1. The lack of a classical NLS suggested that hnRNP H and F might utilize an import receptor distinct from importin α/β , which are the adaptor and import receptors for classical NLS cargo (59, 64). It was previously reported that hnRNP H/F interact with the import receptor Trn1, and hnRNP H with Trn2, and that *in vitro* nuclear import of hnRNP F is mediated by Trn1 (31, 43, 73). Given the high degree of identity between

FIG. 4. Mutations in highly conserved amino acids in the GYR domain disrupt nuclear localization. GFP, GFP-GYR-D, or the indicated GFP-GYR-D mutant GFP fusion proteins shown in Fig. 3 were expressed in HeLa cells and localized by fluorescence microscopy. Mutations or amino acid substitutions incorporated into full-length hnRNP H are indicated. The nuclei are indicated by arrows based on DAPI staining of the same samples. WT, wild type.

hnRNP H and F and the above data indicating the presence of an import signal within the GYR domain, it was predicted that hnRNP H would interact with Trn1 via the GYR domain. To address this possibility, pulldown assays were performed with HeLa cell nuclear extract and GST-tagged wild-type and mutant (Y210A) versions of the GYR domain (194-292) and minimal 194-241 (D) region. An interaction with Trn1 was then assessed by Western blotting. The M9 peptide from hnRNP A1, which interacts with Trn1, and the NLS from the SV40 large T antigen, which binds to importin β via importin α but not to Trn1, were used as positive and negative controls, respectively. As shown in Fig. 5, pulldown of Trn1 was observed with GST-M9, but not GST alone or GST-NLS. Significantly, the GYR domain and the 194-241 Gyr-D region interacted with Trn1 similarly, whereas the interaction was greatly reduced by the Y210A mutation that reduced nuclear localization in cells. Equivalent amounts of the GST proteins were associated with the beads as determined by SDS-PAGE and Coomassie blue staining of a portion of the eluted material (Fig. 5, bottom), supporting the conclusion that the lack of Trn1 signal reflected reduced protein interaction. These results indicate that Trn1 mediates nuclear import of hnRNP H (and likely hnRNP F) through an interaction with the highly conserved region within the GYR domain.

FIG. 5. The hnRNP H GYR domain interacts with transportin 1. (Top) Wild-type and Y210A mutant GST-GYR and GST-GYR-D, GST-NLS, GST-M9, and GST were produced in bacteria, bound to glutathione Sepharose beads, and incubated with HeLa cell nuclear extract. After elution in SDS-PAGE sample buffer, samples were subjected to Western blotting with a Trn1 antibody. (Bottom) One-fifth of the eluted material was subjected to SDS-PAGE, and the gel was stained with Coomassie blue. The positions of size standards are indicated to the left of the gel in kilodaltons. GST proteins (circles) and GST breakdown products (asterisks) are indicated.

FIG. 6. hnRNP H and F are shuttling proteins. myc-tagged versions of hnRNP A1, hnRNP C, hnRNP H, hnRNP F, or the GYR domain of hnRNP H were expressed for 24 h in HeLa cells that were seeded on coverslips. NIH 3T3 cells were then added and allowed to attach, and protein synthesis was blocked with cycloheximide for 1 h. The cells were then fused with PEG 3350, and 1 h later, the cells were fixed and stained with an anti-myc antibody to localize the expressed proteins and DAPI to distinguish human nuclei (arrowheads) and mouse nuclei (arrows). Cells were imaged by phase-contrast microscopy (outline of cell borders indicated by the broken lines).

hnRNP H/F are shuttling proteins. Several hnRNP proteins are restricted to the nucleus (e.g., hnRNP C), whereas others, like hnRNP A1, continuously shuttle between the nucleus and cytoplasm (65, 66). The observations that hnRNP H/F are localized in the cytoplasm of some cells and in some disease states suggest that import of hnRNP H/F may be regulated and/or that the proteins can shuttle. To determine whether they are shuttling proteins, an interspecies heterokaryon assay was employed whereby myc-tagged versions of hnRNP H/F were transfected into HeLa cells, which were then fused to mouse NIH 3T3 cells in the presence of a protein synthesis inhibitor, and the proteins were localized by immunofluorescence microscopy. hnRNP A1 and C were used as positive and negative controls, respectively, and as expected, hnRNP A1

was present in both human and mouse nuclei, whereas hnRNP C was restricted to the HeLa nuclei (Fig. 6). hnRNP H and F proteins were each readily detectable in the HeLa and NIH 3T3 nuclei, indicating that both are shuttling proteins. It was also shown that the GYR domain of hnRNP H supported shuttling, suggesting that the core region of the GYR domain is responsible for both import and export.

Efficient nuclear localization of hnRNP A1, but not hnRNP C, requires ongoing RNA polymerase II (RNAPII) transcription. To determine whether hnRNP H/F proteins have a similar transcriptional requirement for nuclear localization, HeLa cells were transfected with myc-tagged proteins and treated with actinomycin D for 4 h and processed for immunofluorescence. As shown in Fig. 7, hnRNP H and F showed cytoplasmic

FIG. 7. Nuclear localization of hnRNP A1, hnRNP H, and hnRNP F depends upon ongoing polymerase II (Pol II) transcription. myc-tagged versions of hnRNPs A1, C, H, and F were expressed in HeLa cells that were treated for 4 h with the transcription inhibitor actinomycin D (+ ActD) or not treated with actinomycin D (– ActD). Protein localization was determined by immunofluorescence with an anti-myc antibody. In the rightmost column, untransfected HeLa cells were treated with ActD for 4 h or not treated with ActD and subsequently processed for immunofluorescence with an anti-hnRNP H antibody to detect endogenous protein (endog. hnRNP H). The arrows point to cell margins where cytoplasmic staining is evident in treated controls, but not untreated controls.

accumulation in cells treated with actinomycin D, although the cytoplasmic signal was less intense than that observed for hnRNP A1. Cytoplasmic accumulation of endogenous hnRNP H in nontransfected cells was more obvious. These data show that hnRNP H/F proteins behave similarly to hnRNP A1 with respect to transcription-dependent, Trn1-mediated nuclear import and nucleocytoplasmic shuttling properties.

DISCUSSION

The hnRNP family includes ~30 members that were originally thought to simply package mRNA into hnRNP particles, but it is now appreciated that hnRNP proteins have multiple functions and influence mRNA metabolism in profound ways (21, 42). Particular mRNAs are targeted for regulation by the *cis* elements they possess and the specificity of the RNA binding domains contained within hnRNP proteins. This is true of the hnRNP H/F family members where the RNA binding domains have been well studied and shown to interact with G-rich elements (20, 35, 49, 69). In addition to the RNA binding domains responsible for RNA binding specificity, hnRNP proteins feature GRDs that presumably mediate additional functions, since they often engage in protein-protein interactions (13, 50, 71). hnRNP H/F proteins contain two glycine-rich domains, one between RRM2 and RRM3 (GYR) and the other (GY) at the C terminus. While glycine rich, these domains are not conserved and differ from GRDs in other hnRNPs in that they lack the common RGG repeat exemplified by hnRNP A1. We show here that the GYR domain of hnRNP H mediates nuclear import through an interaction with the Trn1 import receptor and is responsible for shuttling properties of hnRNP H and F. This work thus demonstrates an import function of the GYR domain in hnRNP H/F.

GYR domain and nuclear import. Transport into the nucleus occurs through recognition of import signals (NLSs) within protein cargo by import receptors, which bind and release cargo through the action of the RanGTP gradient; cargo is picked up in the RanGDP-rich cytoplasm and is released by the binding of nuclear RanGTP to the import receptor (28,

59). Subsequent to the discovery of the classical basic NLSs that are recognized by importin α and/or β , additional import pathways came to light, including one that utilizes Trn1 as a receptor to recognize a distinct signal from that of importin β .

The canonical Trn1 import signal (M9) was initially described for hnRNP A1, and analogous domains have been described for several other proteins that associate with Trn1/2 (3, 22, 26, 31, 37, 41, 43, 54, 67, 68, 72–74, 76). hnRNP F was previously shown to interact with and be imported by Trn1 (43, 73), and hnRNP H was shown to interact with Trn1/2 (31). The Trn1 cargo import sequences are not highly related, but a consensus sequence of hydrophobic/basic residues followed by R/H/KX₂₋₅PY was recently defined (43). Three of the substitutions within the conserved GYR region used here (R206A, P209A, and Y210A) that disrupt import of hnRNP H and its interaction with Trn1 affected the latter part of the consensus. In addition, R212A reduced import, suggesting that additional residues outside the consensus are required for hnRNP H/F import. This may reflect a requirement for additional positive charge to bind the acidic surface of the Trn1 binding interface. The importance of the upstream hydrophobic consensus may be less stringent, since the P197A substitution in this region was without effect. In support of this, other proteins with M9-like import sequences have basic residues, rather than hydrophobic residues. Differences between hnRNP H and F in the upstream hydrophobic region (aa 194 to 204) might explain the observed cell/tissue-specific differences between the localization of the two proteins.

hnRNP A1 and HuR can also utilize Trn2 as an import receptor, and this may be the case for hnRNP H as well, since it also binds Trn2 (31). However, differences in receptor utilization may exist between hnRNP H and hnRNP F, since an hnRNP F interaction with Trn2 was not detected (31). This may be biologically important, since hnRNP H and F display different degrees of cytoplasmic localization in certain tissues (4, 34, 36, 40, 49).

GYR domain and transcription-dependent nucleocytoplasmic shuttling. Many proteins that continuously move between

the nucleus and cytoplasm contain import and export (nuclear export signal, or NES) signals (28, 59). For example, the HIV Rev protein contains an NLS that interacts with importin β (and others) for import (2, 33, 75) and a separate NES that leads to CRM1-mediated export (23, 25, 80). In contrast, hnRNP A1 and other proteins shuttle between the nucleus and cytoplasm via the M9 sequence alone, which serves as a bidirectional signal (22, 31, 41, 54, 65, 66, 68, 72, 74). Shuttling of M9-containing proteins is also distinct in that cytoplasmic accumulation occurs in the absence of transcription (66). Our data indicate that, like hnRNP A1, the hnRNP H/F import signal also confers bidirectional transport that is sensitive to transcription inhibition.

Despite its role in export, an export receptor that recognizes M9 has yet to be identified. A number of different export receptor-independent mechanisms for M9 export have been suggested that could also pertain to hnRNP H/F. One possibility is that they simply diffuse from the nucleus, which is consistent with the observation that hnRNP A1 is a small protein that passively equilibrates between the nucleus and cytoplasm in early mouse cell embryos (78). This, however, would not explain the requirement for the M9 signal. Alternatively, hnRNP H/F might be exported while complexed with target mRNA, which would explain the cytoplasmic accumulation when transcription is blocked. However, our demonstration that the GYR domain alone is sufficient for shuttling suggests an RNA binding domain-independent mechanism for export; other GYR domain-interacting proteins (see below) might mediate export in this instance. In another possibility based on the results of Becskei and Mattaj (5) for CRM1 export, Trn1 could mediate a reverse transport reaction when hnRNP H/F fail to encounter molecules that anchor them in the nucleus, as was shown for CRM1 export. Last, it is possible that the M9 sequence engages in unknown interactions that promote export. Localization of hnRNP H/F to the cytoplasm suggests additional functions in that compartment, and this notion is supported by the observation that the *Drosophila* homolog Glorund is a translational repressor (39).

Other functions of the GYR domain. The GYR domain is ~75 to 80 aa long, yet ~26 aa from the N terminus of the domain are sufficient to import GFP, and mutations within the very highly conserved core sequence (aa 205 to 213) abolished import. This observation suggests that other portions of the GYR domain may have additional functions and/or influence the activity of the NLS. hnRNP H can interact with itself, hnRNP F, MBNL1, CBP80/20, and PAP (15, 57, 63); thus, it is possible that the GYR domain mediates these or other interactions. Recently, a functional interaction between hnRNP H and Fox2 was reported, and GYR sequences downstream of the NLS were required for a physical interaction with Fox2; this interaction enhances repression of the fibroblast growth factor receptor (FGFR) IIIc exon (50). Thus, a region of the GYR domain distinct from the NLS interacts with Fox2, and this region may also mediate interactions with other proteins.

Posttranslational modifications of RNA binding proteins often influence function. hnRNP H/F are known to be phosphorylated (35, 49), arginine methylated (45), and sumoylated (30, 44). However, the functional significance of these modifications has not been investigated. Proteomic analyses have detected numerous phosphorylated residues throughout hnRNP

H, and thus, RNA binding, subcellular localization, and protein interactions could be affected. Phosphorylation of hnRNP A1 affects nuclear import, as detailed in the introduction (1, 29, 77). Arginine methylation can affect many processes, including subcellular localization (6). Intriguingly, hnRNP H harbors a putative arginine methylation site (GRG) just downstream of the NLS, whereas hnRNP F does not. Selective methylation might underlie the noted localization differences between hnRNP H and F in healthy and diseased tissues (4, 34, 36, 40, 49). Last, sumoylation can affect the localization, activity, or stability of proteins (70). Putative sumoylation sequences of hnRNP H/F lie in the RNA binding domains, and thus in addition to localization, RNA binding could be influenced by SUMO. Future studies are warranted to address these possibilities.

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