A Cellular Protein, hnRNP H, Binds to the Negative Regulator of Splicing Element from Rous Sarcoma Virus*

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Incomplete RNA splicing is a key feature of the retroviral life cycle. This is in contrast to the processing of most cellular pre-mRNAs, which are usually spliced to completion. In Rous sarcoma virus, splicing control is achieved in part through a cis-acting RNA element termed the negative regulator of splicing (NRS). The NRS is functionally divided into two parts termed NRS5 and NRS3, which bind a number of splicing factors. The U1 and U11 small nuclear ribonucleoproteins interact with sequences in NRS3, whereas NRS5 binds several proteins including members of the family of proteins. Among the proteins that specifically bind NRS5 is a previously unidentified 55-kDa protein (p55). In this report we describe the isolation and identification of p55. The p55 binding site was localized by UV cross-linking to a 31-nucleotide segment, and a protein that binds specifically to it was isolated by RNA affinity selection (p55). This article was defrayed in part by the payment of page charges. This paper is available on line at http://www.jbc.org

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* The abbreviations used are: HIV, human immunodeficiency virus; RSV, Rous sarcoma virus; NRS, negative regulator of splicing; snRNP, small nuclear ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; nt, nucleotide(s); SR, serine-arginine rich; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight.

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impairment is caused by the loss of binding of some other factor(s) within this region. In previous studies, an unidentified protein of approximately 55 kDa (termed p55) that is biochemically distinct from the classical SR proteins was shown to bind specifically to NRS5' (10). Using a UV cross-linking assay, we have mapped the p55 binding site to a 31-nucleotide region of NRS5' downstream of the primary SR protein binding site and within the region deleted in the EU8 virus, suggesting a relationship between p55 binding and NRS function. To better understand the significance of p55, we sought to isolate and characterize this protein. Using RNA affinity selection, we have identified p55 as the cellular protein hnRNP H, a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins that are involved in the processing and transport of pre-mRNAs (23). Based on reported activities for hnRNP H, this protein may be involved in NRS-mediated splicing inhibition and/or polyadenylation of viral RNAs.

EXPERIMENTAL PROCEDURES

Plasmid Construction and in Vitro RNA Transcription—RSV DNA fragments were obtained from the Prague C strain (24), and sequence coordinates are as described by Schwartz et al. (25). Plasmids p3ZKXMS, p3ZM3S, p3ZBB, p3ZBBS, and p3ZBBS' were described previously (10, 11). Additional NRS fragments (nucleotides 701–753, 754–797, 797–740, and 740–770) were generated by polymerase chain reaction using primers containing KpnI and XhoI sites and subcloned into these sites in pGEM-3Z (Promega). Primer sequences are available on request. The sequence of all constructs was confirmed by DNA sequencing.

Plasmids were linearized with XhoI (with the exception of p3ZBB, which was linearized with BamHI) and transcribed in vitro using T7 RNA polymerase and [32P]PATP (10, 26). Control RNA was transcribed from PolII-digested pGEM-4Z (Promega). All RNAs were gel-purified before use.

Plasmid p3Z(740–797)x2 was generated by first digesting p3Z(740–797) with KpnI, blunting with T4 DNA polymerase, and then digesting with HindIII. The excised fragment was then subcloned into HindIII-digested p3Z(740–797) to generate the final construct. To produce RNA for affinity selections, this plasmid was linearized with SalI, and large scale in vitro transcription was performed using T7 RNA polymerase (27). Control RNAs for affinity selection were transcribed from PolII-digested pGEM-4Z. All RNAs were trice-labeled with [32P]PATP to monitor yield and coupling to agarose beads (described below).

RNA Affinity Selections—250 pmol of RNA was covalently coupled to agarose beads as described (28). Affinity selections were performed by adding the RNA-agarose beads to 325 μl of reactions containing ~2.65 μg of a 0–65% ammonium sulfate (AS65) fraction of HeLa total cell extract in 12.7 mM Hepes (pH 7.9), 38.5 mM KCl, 20 mM creatine phosphate, 0.4 mM ATP, 3 mM MgCl2, 0.16 mM dithiothreitol, 1.9% glycerol, and 0.08 mM EDTA. After the reactions were incubated at 30 °C for 20 min, the beads were collected, washed four times with 1 ml of 20 mM Hepes (pH 7.9), 100 mM KCl, 5% glycerol, 0.2 mM EDTA, and 4 mM MgCl2 at 4 °C, and resuspended in 30 μl of sample loading buffer. Bound proteins were eluted at 90 °C for 5 min, and half of the supernatant was analyzed on a 10% SDS-polyacrylamide gel. The gel was Coomassie Blue-stained, and bands of interest were excised directly from the gel and subjected to MALDI-TOF analysis (29) by John Leszczk at the Protein Microsequencing and Proteomic Mass Spectrometry Lab at the University of Massachusetts Medical School.

Extract Preparation and Purification of Recombinant Proteins—HeLa S3 cells were grown in spinner flasks, and nuclear extracts were produced as described previously (30). The AS65 fraction used (a gift from L. McNally, Medical College of Wisconsin) was produced as described for the preparation of total HeLa SR proteins (31) and was dialyzed against 20 mM Hepes (pH 7.9), 100 mM KCl, 5% glycerol, and 0.2 mM EDTA before use.

The Q fraction was produced as follows. AS65 extract was adjusted to 2 M NaCl and centrifuged at 213,000 × g for 20 min at 4 °C to extract p55 cross-linking activity from endogenous complexes. The supernatant was collected, concentrated using a centrifugal filter device (Centricon YM-30, Millipore), and separated on a Sephacryl S-200 HR (Amersham Pharmacia Biotech) gel filtration column. Fractions with peak cross-linking activity were pooled, loaded on a prepacked High Q ion exchange column (Econo-Pac, Bio-Rad), and eluted using a salt gradient from 100 to 600 mM KCl. The resulting fractions containing an ~55-kDa cross-linking activity were pooled, diluted with 20 mM Hepes (pH 7.9), 5% glycerol, and 0.2 mM EDTA to approximately 100 mM KCl, and concentrated by centrifugation (Centricon YM-30, Millipore) before use.

To produce recombinant hnRNP H, BL21(DE3)pLysS bacterial cells (Novagen) were transformed with a pET15b vector containing the hnRNP H cDNA (generously provided by D. Black, University of California, Los Angeles), and the protein was purified from inclusion bodies using Ni2+ affinity chromatography (His-Bind Resin, Novagen) as described (32). The recombinant protein was eluted in 20 mM Hepes (pH 7.9), 100 mM KCl, 5% glycerol, and 0.2 mM EDTA containing 250 mM imidazole and dialyzed against 20 mM Hepes (pH 7.9), 100 mM KCl, 5% glycerol, and 0.2 mM EDTA. The protein was concentrated using centrifugal filter devices (Centricon YM-30 and Centricron YM-30, Millipore) according to the manufacturer's instructions. Aliquots were stored at ~50 °C until use. All protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad).

UV Cross-linking—High specific activity RNAs (1010–1016 dpm) were incubated in 25-μl reaction mixtures under modified in vitro splicing conditions (13 mM Hepes (pH 7.9), 100 mM KCl, 20 mM creatine phosphate, 0.4 mM ATP, 3 mM MgCl2, 0.16 mM dithiothreitol, 2% glycerol, 0.08 mM EDTA, and 0.4% mammalian cell and tissue extract Protease Inhibitor Cocktail (Sigma)) with 25 μg of nuclear extract or 2–40 pmol of recombinant hnRNP H for 10–30 min at 30 °C. The samples were spotted onto parafilm at 4 °C and exposed to UV light (254 nm) for 20 min at a distance of ~4 cm, transferred to microcentrifuge tubes containing 20–25 μg of RNase A, and incubated at 37 °C for 15 min. Sample loading buffer was then added, and samples were separated on a 10 or 12% SDS-polyacrylamide gel. Gels were Coomassie Blue-stained to visualize size standards, dried, and analyzed by autoradiography or with a PhosphorImager (Molecular Dynamic Storm 860).

Immunoprecipitations—Protein A-Sepharose beads (Amersham Pharmacia Biotech) were washed in phosphate-buffered saline and suspended at a 50% slurry. 10 μl of slurry was incubated in 500 μl of IP buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100) in the presence or absence of 1 μl of antiserum and rotated at 4 °C for 3 h. Beads were washed three times with 250 μl of IP buffer and resuspended in 100 μl of IP buffer. UV cross-linking was performed as described above, and following RNase treatment, the beads were added to the samples, rotated at 4 °C for 1 h, washed three times with 500 μl of IP buffer, and suspended in 40 μl of sample loading buffer. Bound proteins were eluted by boiling for 5 min, and the supernatants were separated on a 10% SDS-polyacrylamide gel and visualized as described above.

Electrophoretic Mobility Shift Assays—Labeled RNA (2.5 fmol) was incubated at 30 °C for 30 min in 15 μl of 13 mM Hepes (pH 7.9), 70 mM KCl, 20 mM creatine phosphate, 0.4 mM ATP, 3 mM MgCl2, 0.16 mM dithiothreitol, 2% glycerol, 0.08 mM EDTA, and 1 μg of tRNA in the presence or absence of 5 μg of nuclear extract or 10–40 pmol of recombinant hnRNP H. Heparin (Sigma) was then added to a concentration of 5 mM/ml, and the reaction was incubated at 30 °C for an additional 10 min. The completed reaction was separated on a 4% polyacrylamide (29:1) nondenaturing gel. After electrophoresis, the gel was dried and visualized by autoradiography or with a PhosphorImager. For supershifts, assays were performed as described above except that the reactions were initially incubated at 30 °C for 15 min, 1 μl of antiserum was added, and the reactions were incubated an additional 15 min at 30 °C. Antisera against hnRNP H and hnRNP F were generously provided by D. Black. Control antisera (glutathione S-transferase-myelin-basic protein and affinity-purified Exo U) were gifts from R. Fritz and D. Frank, Medical College of Wisconsin.

RESULTS

Localization of the p55 Binding Site—Previous studies established that a number of protein factors in HeLa nuclear extract bind specifically to the 5' portion of the NRS (nucleotides 701–797) (Fig. 1) (10). Although most of these proteins are implicated as being SR proteins (10), the identity of a 55-kDa factor that is biochemically distinct from the SR proteins has remained unclear. The binding site for the SR proteins was localized to nucleotides 715–748 of NRS5' (Fig. 1) (10), but because deletion of this region does not fully eliminate NRS activity (7, 20), other factor(s) that binds within nucleotides 748–797 may contribute to splicing inhibition. Although it was shown that p55 binds NRS5’, its precise binding site was not

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A. The Rous sarcoma virus genome and the negative regulator of splicing. A, the RSV genome is shown above with the relative positions of the long terminal repeats (LTR) and the gag, pol, env, and src genes indicated (not to scale). Alternative splicing is shown by lines between the 5' and 3' splice sites (ss). The location of the NRS is shown within the gag gene and expanded below including sequence coordinates. The overlapping binding sites for the U1 and U11 small nuclear ribonucleoproteins (snRNPs) in the 3' portion of the NRS (nt 913–926) are denoted by a black box. The lightly shaded region indicates the 5' portion of the NRS, and the site where SR proteins were previously shown to bind (nt 715–748) is dark shaded. B, a partial sequence of the purine-rich 5' region of the NRS is shown, and the binding site for p55, as determined in this study, is indicated by brackets.

B. Localization of the p55 binding site to nucleotides 740–770 of the NRS. A, schematic diagram of the RNAs used to localize the p55 binding site within the NRS. 4Z (open box) refers to vector sequences transcribed from pGEM-4Z, and NRS RNAs are shaded. Numbers denote the nucleotides encompassed by the RNAs. The coordinates of NRS, NRS'5, and NRS'3 RNAs are shown in parentheses. The minimal p55 binding site is indicated by a black box. B, UV cross-linking was performed as described under "Experimental Procedures" in 25 μg of HeLa nuclear extract under modified in vitro splicing conditions with the indicated [32P]RNAs (lanes refer to constructs in A). Cross-linked proteins were resolved on a 12% SDS-polyacrylamide gel and visualized with a PhosphorImager. Numbers on the left indicate the positions of 66- and 45-kDa size standards. The relative position of the p55 cross-link is indicated by the bracket. The figure was generated from representative phosphorimages using Adobe Photoshop 5.0.2 and Deneba Canvas 6 on a Power Macintosh 9600/233.

A number of nonspecific RNA-binding proteins were detected when cross-linking was performed with vector RNA (Fig. 2B, lane 1). Consistent with previous studies (10), a specific cross-link of 55 kDa was observed using the full-length NRS and NRS5 but not with NRS3' or vector RNA (Fig. 2B, lanes 1–4). Using the NRS3' variants, we mapped the minimal p55 binding site to nucleotides 740–770, indicating that p55 binds primarily downstream of the SR proteins (Fig. 2B, lanes 5–9). In addition, this region maps within a 42-nucleotide region of the NRS (nt 735–776) previously shown to be functionally important for proper splicing control in a recombinant avian virus (21), further implicating p55 as a potentially important factor in NRS activity. Based on these observations, we sought to purify and identify p55.

Identification of p55 as the Cellular Protein hnRNP H—Because previous data indicated that p55 was present in both nuclear and cytoplasmic HeLa cell extracts (10), we chose to use HeLa total cell extracts as a starting material and follow p55 purification by UV cross-linking. A 0.65% ammonium sulfate precipitation was performed to remove SR proteins, which might interfere with or obscure p55 isolation. Initially this AS65 extract was fractionated biochemically (see "Experimental Procedures"), and enrichment of an ~55-kDa cross-link was observed (data not shown). The predominant protein in this size range was identified by mass spectrometry as a 48-kDa proteolytic fragment of nucleolin (data not shown). Importantly, this protein did not show specific binding to the NRS (data not shown), and since it has not been suggested to participate in any aspect of pre-mRNA splicing (33), we concluded that this protein was unlikely to be biologically relevant to NRS function.

Because it appeared that the specific p55 cross-link was lost in the above purification scheme, we next performed RNA affinity selection directly from AS65 HeLa extracts (Fig. 3). Several proteins were selected with vector RNA, including one that comigrated with the 48-kDa nucleolin fragment (Fig. 3, lane 2). This and other proteins were also detected using a p55-specific NRS RNA, but an additional specific protein of ~55 kDa was also selected (Fig. 3, lane 3). Because this protein was the correct size for p55, it was excised from the gel, digested with trypsin, and subjected to mass spectrometry. Nine of eleven identified masses corresponded to a single protein, and post-source decay analysis unambiguously confirmed this to be the cellular protein, hnRNP H (data not shown).

Antibodies against hnRNP H Specifically Immunoprecipitate...
hnRNP H specifically associates with the NRS5′ region. RNA affinity selection was performed as described under “Experimental Procedures” using *in vitro* transcribed RNAs covalently coupled to agarose beads. The beads were incubated in HeLa AS65 extract and washed extensively. Bound proteins were eluted by boiling, resolved on a 10% SDS-polyacrylamide gel and visualized by Coomassie Blue staining. Bands in the 50–55-kDa range were detected using both RNAs, although at varying intensities, with the exception of an NRS-specific 55-kDa band indicated by an arrow. This protein was excised from the gel and identified by mass spectrometry as hnRNP H.

Numbers on the left indicate the position of size standards (lane M) in kDa. Gels were photographed using an Alphalmager 2000 documentation and analysis system (Alpha Innotech Corp.), and the figure was generated as described in Fig. 2.

**Recombinant hnRNPH Cross-links to the NRS at the p55 Binding Site**—To determine whether hnRNPH interacts directly with the NRS, recombinant hnRNPH was produced in bacteria and utilized in UV cross-linking assays. The recombinant hnRNPH was essentially homogeneous and reacted strongly with anti-hnRNPH antibody (data not shown). Little cross-linking was detected to vector RNA with up to 40 pmol of recombinant hnRNPH (Fig. 6A, lanes 1–3). In contrast, cross-linking to NRS5′ was detected with as little as 2 pmol of recombinant protein, and the cross-link was intensified with increasing hnRNPH concentration (Fig. 6A, lanes 4–7), indicating that hnRNPH binds directly to NRS5′.

It was further predicted that if hnRNPH is p55, cross-linking to the NRS should be dependent on the presence of the p55 binding site (as determined in Fig. 2). To test this, UV cross-linking was performed with recombinant hnRNPH and selected RNAs that were used in Fig. 2 to determine the p55 binding site. Consistent with the results above, cross-linking of hnRNPH was seen with full-length NRS and NRS5′ but not anti-hnRNPH and nuclear extract and not with antiserum alone (Fig. 5, compare lanes 3 to lanes 7–9, and lane 12 to lanes 16–18). Furthermore, although antibodies against hnRNPH supershifted NRS complexes, antibodies against a closely related protein, hnRNPF, did not (Fig. 5, lanes 5 and 14). Taken together, these results indicate that hnRNPH is a component of the NRS5′-specific complex observed with mobility shift assays.

**FIG. 4.** Anti-hnRNPH antiserum immunoprecipitates the p55 cross-link from HeLa AS65 extract. UV cross-linking was performed as described in Fig. 2 using RNAs corresponding to either NRS5′ or the minimal p55 binding site (nt 740–770) in either HeLa AS65 extract or fractionated extract (Q fraction) lacking hnRNPH but enriched for nucleolin (see “Experimental Procedures”). Following cross-linking, samples were incubated with protein A-Sepharose beads (B) either alone or coupled to antibody (Ab). Antiserum against hnRNPH H (H) or the bacterial protein Exo U (U) is indicated. Samples were resolved by SDS-polyacrylamide gel electrophoresis and visualized with a PhosphorImager. Numbers on the left indicate the position of size standards in kDa, and the position of the p55 cross-link is indicated by the bracket. The figure was generated as described in Fig. 2.
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Secondary structure in NRS5′ caused a portion of this RNA to migrate similarly to the hnRNP H-dependent complex, but this pattern was unchanged in the presence or absence of recombinant hnRNP H (Fig. 7B, compare lanes 14 and 17) indicating the protein does not form a complex with this RNA. In addition, no shift was detected using nucleotides 701–753, which do not cross-link p55 or hnRNP H (Fig. 7B, lane 21), but a shift was observed with NRS5′ segments that do cross-link hnRNP H (Fig. 7B, lanes 25 and 30). Taken together, these results confirm that hnRNP H binds directly to the p55 binding site.

**DISCUSSION**

Despite its roles in splicing inhibition, a number of essential splicing factors have been shown to interact with the NRS, including the U1 and U11 snRNPs and several SR proteins. In addition to these factors, a previously unidentified 55 kDa protein (p55) was shown to interact with the NRS by UV cross-linking (10). We became interested in this protein because of its specific binding the 5′ portion of the NRS and because its biochemical properties suggested it was not an SR protein (10). We mapped the binding site for p55 to nucleotides 740–770 of the NRS, downstream of the primary SR protein binding site (nt 715–748). Because this region can support a degree of NRS activity in the absence of the predominant SR protein binding site (7, 20) and is required for maximal NRS-mediated splicing inhibition (21), we sought to identify and characterize this protein.

Through biochemical purification, we identified two proteins of approximately 50–55 kDa that cross-link to nucleotides 740–770 in HeLa cell extracts, only one of which was sequence-specific (data not shown). The nonspecific protein was determined to be a previously described proteolytic fragment of nucleolin containing the RNA binding domains (34). To purify the protein responsible for the specific p55 cross-link, we used RNA affinity and identified it as the cellular factor hnRNP H by mass spectrometry. Three observations support the view that hnRNP H is p55. First, antibodies against hnRNP H immunoprecipitated the p55 cross-link from HeLa cellular extracts. Second, hnRNP H antibodies supershifted complexes that form on NRS5′ in HeLa nuclear extract. Third, recombinant hnRNP H assembled a similar complex and could be cross-linked only to NRS RNAs that contained its binding site, indicating that hnRNP H binds the NRS directly. Furthermore, these hnRNP H-induced complexes migrated identically to those observed in nuclear extract (Fig. 7B, compare lanes 7, 12, 25, and 30 to lanes 5, 10, 23, and 28) suggesting that hnRNP H is solely responsible for these shifts. Collectively, these results indicate that hnRNP H is p55.

hnRNP H is a member of a large group of proteins known as heterogeneous nuclear ribonucleoproteins that bind to RNA polymerase II transcripts and contribute to their maturation to mRNAs (23). The interaction of an hnRNP protein with a viral RNA is not unique. Recently, members of the hnRNP A/B family of proteins were found to bind to an exonic splicing silencer sequence in exon 2 of the HIV-1 tat RNA and shown to be required for splicing inhibition (28). The fact that hnRNP H may be involved in NRS activity might reflect a common role for hnRNPs in viral RNA splicing inhibition. However, the requirement for additional sequences within the NRS (i.e. the U1 site) highlights an important difference between the mechanisms of NRS and HIV tat splicing inhibition.

Although our data do not address function, the significance of hnRNP H in NRS function is suggested by studies of the EU8 recombinant avian leukosis virus (21). This virus, which induces rapid onset B-cell lymphomas through insertional activation of the c- myb gene (22), carries a gag deletion that impairs NRS function (21). This deletion removes nucleotides
735–776 of the NRS, which encompass the hnRNP H binding site. It is tempting to speculate that the loss of hnRNP H binding may result in the NRS defect seen in the EU8 virus. Although we cannot rule out the possibility that other proteins may functionally interact with this region, the specificity of hnRNP H makes it a prime candidate.

In contrast to previously identified NRS binding factors (SR proteins and snRNPs), which have defined roles in pre-mRNA splicing, the role of hnRNP H in cellular RNA metabolism is not clear. Recent reports have implicated hnRNP H in the regulated splicing of at least two genes. Chou et al. (32) have shown hnRNP H to be a component of a neuron-specific enhancer complex that stimulates splicing of the c-src N1 exon. hnRNP H has also been shown to bind an exonic splicing silencer element in exon 7 of the rat β-tropomyosin gene that represses splicing of this exon in nonmuscle cells (35). However, immunodepletion had no effect on the splicing of either an adenoviral (32) or β-globin (35) pre-mRNA, suggesting that hnRNP H is not a general splicing factor. Although hnRNP H is required in both these cases, it is unclear how this protein precisely contributes to either positive or negative splicing regulation. In addition, although these are both examples of tissue-specific alternative splicing, hnRNP H does not exhibit tissue-specific expression in humans (36), rats (37), or mice (35), suggesting that these effects may be mediated via protein-protein interactions. Determination of additional factors that functionally interact with hnRNP H will be important in understanding how this protein acts to modulate pre-mRNA splicing.

hnRNP H is a member of a subfamily of distinct but structurally similar hnRNP proteins that include hnRNP H, hnRNP H', and hnRNP F (36, 38). hnRNP F is 78% identical to hnRNP H (36) and is also a functional component of the c-src splicing enhancer complex (39). Although hnRNP H and hnRNP F may bind as a heterodimer to the c-src enhancer (32), hnRNP F was not detected in hnRNP H-containing NRS5' complexes by supershift assays, suggesting that hnRNP F is not involved in NRS function. hnRNP H', which is 96% identical to hnRNP H, binds a G-rich element downstream of the core SV40 late polyadenylation signal and stimulates 3' end processing (40). Despite their extreme homology, hnRNP H' was not detected by RNA affinity selection with NRS-specific RNAs (data not shown). However, because of the high homology between these two proteins it is unlikely that they possess distinct binding specificities, and the absence of hnRNP H' may reflect a difference in the relative levels of hnRNP H and hnRNP H' in HeLa cells.

How might hnRNP H function in NRS-mediated splicing inhibition? Recent evidence indicates that NRS5' can function as a potent splicing enhancer, which is consistent with the binding of SR proteins to NRS5' (20). However, enhancer activity is not restricted to the primary SR binding region (nt 715–748) and is diffusely localized throughout NRS5' (20).
suggests that hnRNP H may be capable of mediating splicing enhancer activity or augmenting other enhancer activities, consistent with its role in the neural c-src splicing enhancer complex (32). An alternative role for hnRNP H is suggested by the observations that hnRNP H is involved in pre-mRNA 3' end processing (40). Based on the high identity between hnRNP H and hnRNP H', it is likely that the two proteins act similarly in vivo. As discussed previously, the EU8 virus exhibits increased readthrough of viral RNAs into the downstream c-myb gene (22). This readthrough effect is likely caused by the decreased efficiency of viral RNA 3' end processing, which is observed upon deletion of the NRS (41). The loss of the hnRNP H binding site in EU8 suggests a role in polyadenylation. Additionally, because deletion of the hnRNP H binding site appears to lead to
both splicing and polyadenylation defects, it is possible that hnRNP H may participate in the coordination of the splicing and polyadenylation machinery on the viral RNAs. We are currently conducting experiments to explore these possibilities to more clearly define the role of hnRNP H in NRS-mediated splicing inhibition.

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REFERENCES

1. Moore, M. J., Query, C. C., and Sharp, P. A. (1993) in *The RNA World* (Gesteland, R., and Atkins, J., eds), pp. 303–357, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Schwartz, S., Felber, B. K., Benko, D. M., Fenyo, E. M., and Pavlakis, G. N. (1990) *J. Virol.* 64, 2519–2529
3. Purell, D. F., and Martin, M. A. (1993) *J. Virol.* 67, 6365–6378
4. Coffin, J. M. (1996) in *Fields Virology* (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) 3rd Ed., pp. 1926–1936, Raven Press, Ltd., New York
5. Arrigo, S., and Beemon, K. (1988) *Mol. Cell. Biol.* 8, 4858–4867
6. Stoltzfus, C. M., and Fogarty, S. J. (1989) *J. Virol.* 63, 1669–1676
7. McNally, L. M., and McNally, M. T. (1999) *Mol. Cell. Biol.* 19, 69–77
8. Hihet, C. S., Gentarek, R. R., and Beemon, K. L. (1999) *RNA* 5, 333–343
9. Gentarek, R. R., McNally, M. T., and Beemon, K. (1993) *Genes Dev.* 7, 1926–1936
10. McNally, L. M., and McNally, M. T. (1996) *J. Virol.* 70, 1163–1172
11. McNally, L. M., and McNally, M. T. (1999) *J. Virol.* 73, 2385–2393
12. Hall, S. L., and Padgett, R. A. (1984) *J. Mol. Biol.* 180, 357–365
13. Kolosova, I., and Padgett, R. A. (1997) *RNA* 3, 227–233
14. Tarn, W. Y., and Steitz, J. A. (1996) *Cell* 84, 801–811
15. Kohtz, J. D., Jamison, S. F., Will, C. L., Zuo, P., Luhrmann, R., Garcia-Blanco, M. A., and Manley, J. L. (1994) *Nature* 370, 119–124
16. Fu, X. D. (1995) *RNA* 1, 663–680
17. Manley, J. L., and Tacke, R. (1996) *Genes Dev.* 10, 1569–1579
18. Reed, R. (1996) *Curr. Opin. Genet. Dev.* 6, 215–220
19. Wang, J., and Manley, J. L. (1997) *Curr. Opin. Genet. Dev.* 7, 205–211
20. McNally, L. M., and McNally, M. T. (1998) *Mol. Cell. Biol.* 18, 3103–3111
21. Smith, M. R., Smith, R. E., Dunkel, I., Hou, V., Beemon, K. L., and Hayward, W. S. (1997) *J. Virol.* 71, 6534–6540
22. Jiang, W., Kanter, M. R., Ramsay, R. G., Beemon, K. L., and Hayward, W. S. (1997) *J. Virol.* 71, 6526–6533
23. Dreyfuss, G., Matunis, M. J., Pinnol-Roma, S., and Burd, C. G. (1993) *Annu. Rev. Biochem.* 62, 289–321
24. Meric, C., and Spahr, P. F. (1986) *J. Virol.* 60, 450–459
25. Schwartz, D. E., Tizard, R., and Gilbert, W. (1983) *Cell* 32, 853–869
26. Melton, D. A., Krieg, P. A., Rabaglisti, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) *Nucleic Acids Res.* 12, 7035–7056
27. Beckler, G. S. (1993) *Promega Notes* 39, 12–16
28. Caputi, M., Mayeda, A., Krainer, A. R., and Zahler, A. M. (1999) *EMBO J.* 18, 4060–4067
29. Chaurand, P., Luestersenkirchen, F., and Spengler, B. (1999) *J. Am. Soc. Mass Spectrom.* 10, 91–103
30. Dignam, J. D., Lebovitz, R. M., and Roeder, R. D. (1983) *Nucleic Acids Res.* 11, 1473–1489
31. Zahler, A. M., Lane, W. S., Stolk, J. A., and Roth, M. B. (1992) *Genes Dev.* 6, 837–847
32. Chou, M., Rook, N., Turk, C. W., and Black, D. L. (1999) *Mol. Cell. Biol.* 19, 69–77
33. Giain, H., Sicala, J., Koger, S., and Bovet, P. (1999) *J. Cell Sci.* 112, 761–772
34. Sapp, M., Richter, A., Weisheart, K., Caizergues-Ferrer, M., Amalric, F., Wallace, M. O., Kirstein, M. N., and Olsen, M. O. J. (1989) *Eur. J. Biochem.* 179, 541–548
35. Chen, C. D., Kobayashi, R., and Helfman, D. M. (1999) *Genes Dev.* 13, 593–606
36. Honore, B., Rasmussen, H. H., Vorum, H., Dejgaard, K., Liu, X., Gromov, P., Madisen, P., Gesser, B., Tommerup, N., and Celis, J. E. (1995) *Eur. J. Biochem.* 270, 28780–28789
37. Holzmann, K., Kurose, T., Gerner, C., Grimm, R., and Stuermer, G. (1997) *Eur. J. Biochem.* 244, 479–486
38. Matunis, M. J., Xing, J., and Dreyfuss, G. (1994) *Nucleic Acids Res.* 22, 1059–1067
39. Moc, H., Chan, R. C., and Black, D. L. (1995) *Genes Dev.* 9, 2659–2671
40. Bagga, P. S., Arhin, G. K., and Wilusz, J. (1998) *Nucleic Acids Res.* 26, 5343–5350
41. Miller, J. T., and Stoltzfus, C. M. (1992) *J. Virol.* 66, 4242–4251