The 3′ untranslated region of human vimentin mRNA interacts with protein complexes containing eEF-1γ and HAX-1

May Al-Maghrébi, Hervé Brulé, Marina Padkina, Carrie Allen, W. Michael Holmes and Zendra E. Zehner*

Department of Biochemistry and Molecular Biophysics and the Massey Cancer Center, 1 Institute for Structural Biology and Drug Discovery and 2 Department of Microbiology and Immunology, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, VA, USA and 3 Laboratory of Biochemical Genetics, Biological Institute, St Petersburg State University, Russia

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ABSTRACT

Previously, we have shown that the vimentin 3′ untranslated region (3′UTR) contains a highly conserved region, which is sufficient for the perinuclear localization of a reporter mRNA. This region was shown to specifically bind protein(s) by band shift analyses. UV-cross-linking studies suggest these proteins are 46- and 35-kDa in mass. Here, we have used this sequence as ‘bait’ to isolate RNA binding proteins using the yeast three-hybrid method. This technique relies on a functional assay detecting bona fide RNA–protein interaction in vivo. Three cDNA isolates, HAX-1, eEF-1γ and hRIP, code for proteins of a size consistent with in vitro cross-linking studies. In all cases, recombinant proteins were capable of binding RNA in vitro. Although hRIP is thought to be a general mRNA binding protein, this represents an unreported activity for eEF-1γ and HAX-1. Moreover, HAX-1 binding appears to be specific to vimentin’s 3′UTR. Both in vivo synthesized eEF-1γ and HAX-1 proteins were ‘pulled out’ of HeLa whole cell extracts by binding to a RNA affinity column comprised of vimentin’s 3′UTR. Moreover, size-fractionation of extracts results in the separation of large complexes containing either eEF-1γ or HAX-1. Thus, in addition to their known functions, both eEF-1γ and HAX-1 are RNA binding proteins, which suggests new roles in mRNA translation and/or perinuclear localization.

INTRODUCTION

The 3′ untranslated region (3′UTR) of many eukaryotic mRNAs has been implicated in a variety of cellular processes, which include mRNA processing, polyadenylation, stability, localization and translational regulation. In each case, functional activity appears to require a specific interaction between RNA element(s) and one or more RNA binding protein. For example, AU- or CU-rich elements bind protein(s) that affects the stability of myc or globin mRNAs, respectively (1,2). In hepatocytes, cCp2 increases type 1 collagen protein levels by binding to the 3′UTR and increasing collagen mRNA stability (3). Other proteins bind elements which regulate the site of translation. For example, differentiation control elements (DICE) within the 3′UTR of 15 lipoxygenase mRNA bind hRNPs, which mediate translational silencing during erythrocyte maturation (4). Nanos represses the translation of maternal hunchback mRNA in the posterior portion of the developing Drosophila embryo by altering the state of mRNA polyadenylation (5). In chicken fibroblasts, translation of β-actin mRNA is localized to the lamellipodia where its protein product is required to lend support to the growing cellular extension (6,7). On the other hand, β-actin is localized to the dendritic process in neurons, suggesting that the same mRNA may move to a different cellular location depending on the cell-type and protein(s) with which it interacts (8). Thus, RNA–protein interactions are crucial for maintaining proper RNA metabolism.

To date, sequences that are required for mRNA localization have been found within the 3′UTR of their respective mRNAs. Such sequences have been termed zipcodes, since they function to direct mRNAs to their final cellular destination (9). While β-actin mRNA is localized to the cellular periphery, vimentin and γ-actin mRNAs have been found to be perinuclearly localized (6,10–12). In the case of vimentin, perinuclear localization may enhance filament assembly, since it takes the association of 32 chains to build an intermediate filament (IF) (13). Pulse–chase experiments indicate that the pool of soluble vimentin in the cell is actually the coiled–coil tetramer, an important intermediate in assembly of the final filament (14). Thus, there is little free vimentin monomer in the fibroblast cytoplasm. Recent studies indicate that partially assembled vimentin subunits, referred to as dots and squiggles, and representing dimers or more likely tetramers, are moved on detyrosinated, stable microtubules (Glu MTs)
from the perinuclear to the peripheral region of the cell where they are thought to add on to the assembling IF network (15,16). Microinjection of affinity purified antibodies specific for Glu tubulin but not Tyr tubulin result in the collapse of vimentin to the perinuclear region. A similar result is obtained upon microinjection of kinesin antibodies suggesting a role for a kinesin motor in mediating IF movement on Glu MTs (16–19). Based on these results a co-translational mechanism has been proposed for IF assembly, suggesting that perinuclear localization of vimentin mRNA might be an important component of network assembly (20). In support of this hypothesis, it has been shown that the misdirection of vimentin mRNA to the cell periphery results in altered cell morphology and motility (21). In addition, perinuclear localization has been shown to be dependent on IF content (22). In order to better understand what contributes to the regulation of vimentin synthesis, we have initiated a study of the 3’ UTR of vimentin mRNA.

Previously, we have shown that a highly conserved RNA element within vimentin’s 3’ UTR (from position –37 to –149 downstream of the stop codon) is capable of localizing a green fluorescent protein (GFP) reporter mRNA to the perinuclear region (22). This region forms a bifurcated, stem–loop secondary structure in solution (23). Here, we have shown that a sub-region interacts specifically with proteins (of ~46- and 35-kDa) in HeLa whole cell or cytoplasmic extracts. Using the yeast three-hybrid method we have isolated and identified three human candidate proteins to be HAX-1 (HS1-associated protein X-1), the eukaryotic elongation factor 1-gamma (eEF1-γ) and the human rev interacting protein (hRIP). A variety of in vitro and in vivo studies confirm these proteins do bind to the 3’UTR of vimentin mRNA and HAX1 binding appears to be specific. Finally, we discuss the possible implications of this interaction for regulating vimentin mRNA translation, synthesis, and/or filament formation.

MATERIALS AND METHODS

Screening of a HeLa cDNA library in yeast via the three-hybrid method

A hybrid RNA construct (Y-pIII/Mp17) containing a modified version of vimentin’s 3’ UTR (from –54 to –123 downstream of the stop codon) (as discussed under Results) was constructed by inserting the desired vimentin DNA fragment into a unique SmaI site downstream of the phage MS2 DNA in the pIII/Mp17 vector, which contains the URA3 gene as a selectable marker (gift from Dr Marvin Wickens, University of Wisconsin) (24,25). A HeLa cDNA library fused to the Gal4 activation domain (AD) in the pGAD-GH plasmid, which carries the LEU2 gene as a selectable marker, was a gift from Dr William Marzluff, University of North Carolina. The Y-pIII/Mp17 and HeLa cDNA library plasmids were sequentially transformed using the lithium acetate method into the yeast L40 coat strain (24). This strain contains both histidine (HIS3) and leucine (LEU2) selectable markers, was a gift from Dr William Marzluff, University of Wisconsin) (24,25). A HeLa cDNA library was constructed by inserting the desired vimentin DNA downstream of the stop codon (as discussed under Results). cDNA plasmids were isolated from yeast using glass beads and used to transform HB101 Escherichia coli cells by electroporation. Plasmids were purified by the alkaline-lysis method and DNA inserts sequenced by the dideoxy method. Sequences were searched against the GCG database for protein identification.

Preparation of recombinant proteins

cDNA clones of interest were cloned into the pET-16b vector (Novagen) using PCR and specific primers to produce His-tag recombinant proteins via protocols recommended by the manufacturer. DNA inserts were sequenced to verify reading frame. Bacteria were grown at 37°C to a density of 0.5 at A600nm, followed by IPTG induction at 1 mM for 3–4 h. Recombinant proteins were purified as described by Qiagen for His-tag protein purification under native conditions. Eluted proteins were analyzed by 12% SDS-PAGE and stained with Coomassie blue. Purified recombinant proteins were stored at –20°C in 50% glycerol.

Preparation of HeLa extracts

HeLa whole cell extracts (WCE) were prepared from HeLa cell cultures as previously described (23,26,27). HeLa cytoplasmic extract was prepared as described (28). Protein concentration was determined by the Bradford assay.

Preparation of RNA transcripts and RNA band shift assays

Subcloning of various vimentin 3’UTR fragments to use as RNA templates was done using PCR and specific primers as previously described (23). Regions of RNA selected for the various assays are identified by their position relative to the stop codon, i.e., from –37 to –147 downstream of the stop codon is represented as 37/147. In vitro transcripts were prepared using T7 RNA polymerase according to established protocols in the presence or absence of [α-32P]NTPs (23). Non-specific RNA was synthesized from the brome mosaic virus (BMV) 3’UTR (gift from Dr Catherine Florentz, IBMC, Strasbourg, France) or from the 3’UTR of γ-actin (160/396). RNA band shift assays were done as previously noted using the amounts indicated in the figure legends (23). In competition band shift assays, 32P-labeled RNA was first
incubated with increasing concentrations of either specific or non-specific, unlabeled RNA competitors. This was followed by the addition of 3 μg or 50 ng of HeLa WCE or recombinant protein, respectively. In band shift assays with specific antibodies for eEF-1γ (gift from Dr G. Janssen), or HAX-1 (Transduction Laboratories, Lexington, KY) increasing concentrations of each antibody was added either before or after RNA–protein complex formation. Either order of addition yielded the same result. Reactions were carried out as cited above, but at room temperature.

UV cross-linking analysis

32P-labeled vimentin RNA (37/147) (300 000 d.p.m.) was incubated with HeLa WCE (20 μg) as mentioned above for band shift assays. The sample was exposed to UV light (254 nm) at a distance of 5 cm for 15 min on ice, and unbound RNA was removed as described in the legend. To Figure 2. UV cross-linking studies were repeated using either 32P-labeled vimentin or 32P-labeled γ-actin RNA (160/396) in the presence of a 50-fold excess of unlabeled, non-specific γ-actin RNA.

Western Blotting

Cell extracts or recombinant proteins were resolved on 12% SDS–PAGE and transferred to nitrocellulose membrane by electrophoresis at 100 or 150 V for 1 or 0.75 h, respectively, at 4°C. The membrane was blocked with 3% non-fat milk for 1 h at room temperature, followed by hybridization with primary specific antibody at the dilution recommended by the supplier in 0.6% non-fat milk, overnight, at 4°C with shaking. The membrane was washed 3× with PBS–Tween-20 for 10 min each, 1× with PBS alone, and hybridized with the appropriate HRP-secondary antibody (1:20 000 dilution) as recommended by Amersham Life Sciences, washed 3× in PBS–TWEEN-20, 1× in PBS alone, and detected using a ECL kit (Amersham/Pharmacia).

Analytical RNA affinity purification of RNA-binding proteins

Unlabeled RNA transcript (37/147) was pre-heated at 65°C in 0.1× SSC prior to the addition of 150 pmol of synthetic, biotinylated oligonucleotide (5′-CGTTAAGAACTAGAGCTTATTCC-3′), complementary to the 3′-end of the 37/147 transcript (from region –127 to –147), but downstream of the required protein binding domain. The mixture was allowed to cool to room temperature. The annealed mix was incubated with pre-washed streptavidin paramagnetic particles (SA-PMP) (Promega) at room temperature for 10 min. The immobilized RNA transcript was washed twice with 0.5× SSC and then incubated with HeLa WCE (277 μg) on ice for 15 min in the presence of binding buffer (25 mM Tris–HCl, pH 7.5, 5 mM MgCl2 and 150 mM KCl). The immobilized RNA and its bound proteins were separated from the crude cell extract on a magnetic stand. The RNA–protein complex bound to the SA-PMP was washed twice with the binding buffer and proteins eluted first with water and then by boiling for 5 min in water, in which case the beads were removed by centrifugation prior to gel loading. The content of the various fractions was resolved by 12% SDS–PAGE and transferred to nitrocellulose membrane for western blotting. A synthetic RNA oligonucleotide (from region –127 to –147) and complementary to the biotinylated DNA oligonucleotide was used as a negative control.

Size fractionation of HeLa cytoplasmic extracts

HeLa cytoplasmic extract (equivalent to a S-100) containing 0.25% NP-40 was size-fractionated on a Superose 6 (prep grade from Amersham Pharmacia Biotech AB) column (16 mm × 70 cm) using HPLC at a flow rate of 1 ml/min and 70 fractions of 2 ml each were collected. The sample and running buffer contained 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 10% glycerol. Following fractionation, glycerol was added to a final concentration of 40% and the column fractions were stored at −20°C. Molecular weight markers were used to calibrate the column as noted. RNA band shift assays were conducted on each fraction (10 μl) as described above. Western blots with eEF-1β, eEF-1γ and eEF1-δ, (a gift from Dr G. Janssen), and HAX-1 (Transduction Laboratories) antibodies were carried out on 12–14 μl of each fraction as mentioned previously. Only results from those fractions that contained protein (antigen) are shown.

RESULTS

In a previous study, band shift assays with 32P-labeled RNA synthesized from various regions of vimentin’s 3′UTR suggested that a RNA binding domain extended from position –61 to –114 downstream of the stop codon (23). Here, we verify that the binding domain (–37 to –123) is sufficient for protein binding (Fig. 1A). A slightly larger region (–39 to –147) has been shown both necessary and sufficient for the perinuclear localization of a GFP reporter mRNA (22). The specificity of this RNA–protein interaction was confirmed by competition band shift assays (Fig. 1B). The addition of a 200-fold excess of unlabeled specific (self) RNA led to a 90% reduction in protein binding, whereas a similar amount of excess BMV (non-specific) RNA had little effect.

UV cross-linking studies confirm the binding of a 46-kDa protein(s) as well as a 35-kDa protein(s) (Fig. 2A). This experiment was repeated in the presence of a 50-fold excess of non-specific, γ-actin RNA (160/396) with 32P-labeled vimentin RNA (37/147) (lanes 2 and 4) as well as 32P-labeled RNA prepared from the 3′UTR of γ-actin mRNA (160/396) (lanes 1 and 3). This region of γ-actin’s 3′UTR is similar in length to vimentin’s 3′UTR but does not contain a known RNA binding site (Fig. 2B). Lanes 1 and 2 verified that all free RNA was totally removed by RNase treatment, thus the bands detected in lane 4 were due to the cross-linking of 32P-labeled RNA to protein and not the inadequate removal of excess 32P-labeled RNA. In addition, the same result was obtained in the presence of excess non-specific γ-actin RNA (Fig. 2B) as in its absence (Fig. 2A) attesting to the specificity of RNA–protein binding in lane 4. Previously, when RNA was synthesized with [32P]ATP, a 46-kDa protein (doublet) was noted (23). Here, we synthesized the RNA transcript with [32P]UTP, which resulted in the detection of an additional protein of ~35-kDa. A repeat of this experiment with [32P]ATP labeled RNA gave only the 46-kDa protein band as noted previously (data not shown).
Further analysis of these clones was not warranted, since the sequence for the U5 snRNP protein (100-kDa). It was felt that the U5 protein has not the appropriate molecular weight, is a nuclear protein, and is known to bind to short stretches of U residues, of which there are several within vimentin’s RNA binding domain. Therefore, we felt this binding was not specific to vimentin mRNA. Similarly, the clones KIAA0098 (encodes the human counterpart of the mouse chaperonin containing TCP-1 gene) and KIAA0207 (coding for a human growth factor receptor-bound protein Grb10) were put aside since the size of their encoded protein was larger than the desired 46- or 35-kDa target protein, although a faint band around 60-kDa (Fig. 2B, lane 4) suggests there may be an additional RNA binding protein of this molecular weight, which awaits future studies. Of the remaining candidates, 25% (8 clones) encoded a protein referred to as HAX-1 (35-kDa), 9.4% (3 clones) encoded a 50-kDa protein identified as eEF-1γ (30,31) and 3% (only 1 clone) encoded hRIP (32-kDa), a human REV-interacting protein (G.Chinnadurai, personal communication). The remaining 25% (8 clones) were all single isolates, of unknown function and product size, which were excluded from further analysis at this time. Due to their apparent size, cellular location, and possible functional importance, the three clones encoding HAX-1, eEF-1γ and hRIP were selected for further analysis.

Yeast three-hybrid screening

Since the RNA binding domain contained five U residues (at position –95 to –99), which could be a possible transcription termination site for yeast Pol III, the sequence of this region was changed to UUAUU. A band shift assay confirmed that the single A to U base mutation had little effect on protein binding (data not shown). Thus, a mutated, slightly smaller RNA binding domain (±54 to ±123) was inserted downstream of the MS2 RNA element and used as bait for detecting RNA binding proteins in vivo via the yeast three-hybrid method (24,25).

Yeast transformants (4 × 10⁹) were screened, and 32 positive clones were identified based on their ability to grow and produce only white colonies on media minus histidine in the presence of 5 mM 3-AT and confirmed for their ability to activate the second reporter gene β-gal. These controls serve to separate the true positive from background false positives as discussed further in Materials and Methods. From DNA sequencing the content of the various cDNA inserts were identified as follows (Table 1). Four clones contained the sequence for the U5 snRNP protein (100-kDa). It was felt that further analysis of these clones was not warranted, since the U5 protein has not the appropriate molecular weight, is a nuclear protein, and is known to bind to short stretches of U residues, of which there are several within vimentin’s RNA binding domain. Therefore, we felt this binding was not specific to vimentin mRNA. Similarly, the clones KIAA0098 (encodes the human counterpart of the mouse chaperonin containing TCP-1 gene) and KIAA0207 (coding for a human growth factor receptor-bound protein Grb10) were put aside since the size of their encoded protein was larger than the desired 46- or 35-kDa target protein, although a faint band around 60-kDa (Fig. 2B, lane 4) suggests there may be an additional RNA binding protein of this molecular weight, which awaits future studies. Of the remaining candidates, 25% (8 clones) encoded a protein referred to as HAX-1 (35-kDa) (29), 9.4% (3 clones) encoded a 50-kDa protein identified as eEF-1γ (30,31) and 3% (only 1 clone) encoded hRIP (32-kDa), a human REV-interacting protein (G.Chinnadurai, personal communication). The remaining 25% (8 clones) were all single isolates, of unknown function and product size, which were excluded from further analysis at this time. Due to their apparent size, cellular location, and possible functional importance, the three clones encoding HAX-1, eEF-1γ and hRIP were selected for further analysis.

Analysis of binding specificity using control and mutant RNAs in the yeast three-hybrid system

The yeast three-hybrid system was used to further analyze the RNA binding specificity between HAX-1, eEF-1γ, and control and mutant RNAs as recommended (24). Since only one plasmid encoding hRIP was obtained in the initial screen, we excluded this cDNA from this analysis. Various MS2 hybrid RNA plasmids containing either vimentin’s RNA strand (37/123 referred to as MS2-Vim), the iron regulatory element (IRE-MS2), or the MS2 RNA alone (MS2) were co-transfected into yeast strain L40 coat with either the HAX-1,

Figure 2. Analysis of RNA binding protein activity by UV cross-linking. (A) 32P-labeled RNA (37/147) (300 000 d.p.m.) was incubated with 20 μg of HeLa WCE. Following exposure to UV light at 254 nm for 15 min, non-cross-linked RNA was removed by digestion with RNase A (6 U) and T1 (1.5 U) for 2 h at 37°C. Proteins were separated on a 10% SDS–PAGE gel. The position of migration for several molecular weight markers is indicated by arrowheads. (B) Cross-linking studies were repeated in the presence of a 50-fold excess of non-specific γ-actin RNA (160/396) with 32P-labeled vimentin RNA (37/147) (lanes 2 and 4) or 32P-labeled γ-actin RNA (160/396) (lanes 1 and 3). HeLa WCE (20 μg) was incubated with the respective RNAs in lanes 2 and 4 whereas RNA alone was analyzed in lanes 1 and 3. The position of migration for several molecular weight markers is shown as in (A).

Figure 1. RNA band shift assay with HeLa WCE. (A) Twelve femtomoles of 32P-labeled RNA from the 3’UTR of human vimentin mRNA from position –37 to –123 downstream of the stop codon (37/123) was incubated alone (lane 1) or with increasing concentrations, 1 μg (lane 2), 2 μg (lane 3) or 4 μg (lane 4), of HeLa WCE. RNA–protein complexes were separated from free RNA on a 5% polyacrylamide gel, dried, and exposed to film overnight at –70°C. (B) 32P-labeled RNA as in (A) was incubated with 4 μg of HeLa WCE and increasing concentrations of unlabeled specific 37/123 (squares) and non-specific BMV (circles) competitor RNA. Data was quantified on a phosphoimage analyzer (Molecular Dynamics) and the percent of shifted material is plotted versus the fold-excess of cold RNA added.
Table 1. Tally of cDNA clones isolated by the yeast three-hybrid screening method

<table>
<thead>
<tr>
<th>cDNA Isolates</th>
<th>No. of isolates (%)</th>
<th>Protein Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAX-1</td>
<td>8 (25%)</td>
<td>Nuclear envelope, ER, mitochondrial protein (35 kDa)</td>
</tr>
<tr>
<td>Human mRNA for KIAA0098</td>
<td>5 (14.2%)</td>
<td>Human counterpart of mouse chaperonin containing TCP-1 gene (&gt;60 kDa)</td>
</tr>
<tr>
<td>U5 snRNP</td>
<td>4 (12.5%)</td>
<td>Pre-mRNA splicing factor (100 kDa)</td>
</tr>
<tr>
<td>eEF-1γ</td>
<td>3 (9.4%)</td>
<td>Eukaryotic elongation factor-1 (50 kDa)</td>
</tr>
<tr>
<td>Human mRNA for KIAA0207</td>
<td>3 (9.4%)</td>
<td>Human growth factor receptor-bound protein Grb10 (66.5 kDa)</td>
</tr>
<tr>
<td>hRIP</td>
<td>1 (3%)</td>
<td>Human Rev-interacting protein (32 kDa)</td>
</tr>
<tr>
<td>Single isolates</td>
<td>8 (25%)</td>
<td>Single isolates with no known function</td>
</tr>
</tbody>
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The results would appear that there is some flexibility in the RNA sequence that eEF-1γ can bind. Also, due to the requirement to lower the 3-AT concentration to obtain growth, binding is not as strong as HAX-1 to vimentin’s 3’UTR or IRP to IRE.

**Analysis of recombinant eEF-1γ, HAX-1 and hRIP binding to vimentin’s 3’UTR**

The aforementioned analysis in yeast demonstrates the ability of these fusion proteins and hybrid RNAs to interact in vivo, which correlates to how these proteins might function in the eukaryotic cell. We next addressed whether or not these proteins minus the Gal4 AD or the RNA sequence minus the MS2 RNA could bind in vitro. In order to determine if these cDNAs encode vimentin RNA binding proteins in vitro, Histag recombinant proteins were expressed in bacteria and purified to apparent homogeneity. Protein purity was assessed by Coomassie blue staining of SDS–polyacrylamide gels. In each case, single protein bands of the appropriate molecular weight were obtained (data not shown).

32P-labeled RNA (37/123) was incubated with HeLa WCE or purified recombinant eEF-1γ and the RNA–protein complexes resolved via band shift assays (Fig. 4A, lane 2 or 3, respectively). It is noteworthy that the migration of the RNA–recombinant protein complex using purified eEF-1γ is significantly faster than the RNA–protein complex with HeLa WCE. This result suggests that other proteins in addition to eEF-1γ are present in the protein complex with HeLa WCE. The specificity of protein binding was further assessed by a competition assay in the presence of unlabeled specific RNA (Fig. 4B). As little as a 5-fold excess of specific RNA reduced binding significantly. By 50-fold excess, little shifting activity remained. However, protein binding in the presence of BMV RNA was similarly affected suggesting that eEF-1γ binding is not specific to vimentin’s 3’UTR.

To determine if eEF-1γ is indeed part of the RNA–protein binding complex, its specific antibody (anti-eEF-1γ) was used to further probe the protein content of RNA band shift assays. Pre-incubation of increasing amounts of anti-eEF-1γ with purified recombinant eEF-1γ protein (Fig. 4C) results in partial ablation of RNA band shift activity. Band shift assays with HeLa WCE yield similar results (data not shown). This suggests that eEF-1γ is part of the RNA–protein binding complex assembled in vitro from purified components. However, when eEF-1γ is bound to the RNA it is not accessible to antibody binding perhaps due to its conformation. Instead, antibody recognition must compete with RNA for protein binding, since an ablation of binding is seen rather than a supershift. The addition of increasing concentrations of
prior ablation of RNA–protein interaction must be due to a specific recognition between antibody and antigen and not due to a non-specific interaction between antibody and any protein.

Similar studies were conducted with HAX-1 and hRIP. Purified, bacterially-expressed, recombinant HAX-1 was also
capable of binding to RNA in the band shift assay (Fig. 5A). The addition of increasing amounts of excess unlabeled, specific vimentin RNA versus non-specific RNA suggested that HAX-1 binding was specific (Fig. 5B). However, additional RNA band shift assays with anti-HAX-1 added either prior to or after RNA–protein incubation yielded no effect on RNA band shift ability with either purified recombinant HAX-1 protein or with HeLa WCE (data not shown). Western blots confirmed that the bacterially-expressed recombinant protein was indeed HAX-1. From these studies, it was concluded that either anti-HAX-1 is not binding to the same site on RNA as the HAX-1 protein or the antibody cannot recognize HAX-1 when bound to RNA, since neither ablation nor a supershift of RNA–protein complexes could be detected.

On the other hand, recombinant hRIP binding was judged to not be specific to vimentin because of the following results (data not shown). In band shift assays protein–RNA interaction was competed with any RNA. The addition of anti-hRIP either prior to or following protein addition had no effect on band shift activity. Since only one cDNA plasmid encoding hRIP was isolated in the yeast three-hybrid screen, it was concluded that hRIP binding might not be specific to vimentin mRNA in fitting with hRIP’s putative role in nuclear RNA export. hRIP was isolated in a yeast two-hybrid screen using HIV-1 Rev as bait. It is thought to bind to the basic domain of HIV Rev, which is involved in binding with the RRE, and thus may indirectly contribute to RNA export (G.Chinnadurai, personal communication). Due to these results, it was felt that further studies on hRIP did not seem warranted at this time.

eEF-1γ and HAX-1 as present in HeLa WCE interact with the vimentin 3’UTR

In order to confirm that in vivo synthesized eEF-1γ and HAX-1 is able to recognize and bind the vimentin 3’UTR, an RNA affinity column was prepared. A biotinylated oligonucleotide, which is complementary to the 3’-end of the RNA transcript (from region –125 to –147) extending downstream of the required RNA binding domain, was used to immobilize the RNA binding domain on SA-PMP. This RNA affinity column was used to ‘pull out’ RNA-binding proteins from HeLa WCE. The identity of the RNA-binding proteins was verified by western blots with antibodies specific to eEF-1γ (Fig. 6) or HAX-1 (Fig. 7). Recombinant eEF-1γ (Fig. 6A, lane 7) or HAX-1 (Fig. 7A, lane 7) was included as a positive control for antibody specificity. The protein content of the starting WCE was assessed in lane 6. The binding reaction was carried out with above saturation conditions as indicated by the continued presence of eEF-1γ or HAX-1 in the unbound fraction (Figs 6A and 7A, lane 3). Unbound proteins were removed by washing two times with binding buffer (Figs 6A and 7A, lanes 4 and 5, respectively). Bound proteins were either eluted with water (Figs 6A and 7A, lane 1) or boiled in SDS-loading buffer and the magnetic beads removed prior to loading the supernatant on the gel (Figs 6A or 7A, lane 2).
eEF-1γ most protein was eluted with the water wash (Fig. 6A, lane 1). Only a small amount remained associated with the SA-PMP and was removed by boiling in the presence of SDS (Fig. 6A, lane 2). On the other hand, for HAX-1, boiling in the SDS-loading buffer was required to remove HAX-1 from the RNA affinity column (Fig. 7A, lane 2). In addition, a doublet was noted with in vivo synthesized HAX-1 suggesting that eukaryotic HAX-1 might be a target for post-translational modification, which is not duplicated with the bacterially-expressed protein.

To show that eEF-1γ and HAX-1 binding is dependent on the presence of vimentin’s RNA minimal binding domain, the ‘pull-out’ assay was repeated in the presence of only the SA-PMP and was removed by boiling in the presence of SDS (Fig. 6A, lane 2). On the other hand, for HAX-1, boiling in the SDS-loading buffer was required to remove HAX-1 from the RNA affinity column (Fig. 7A, lane 2). In addition, a doublet was noted with in vivo synthesized HAX-1 suggesting that eukaryotic HAX-1 might be a target for post-translational modification, which is not duplicated with the bacterially-expressed protein.

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The first peak of RNA band shift activity co-eluted with fractions containing eEF-1γ as detected by western blot (Fig. 8C). The mobility of this peak was indicative of a large complex (>200-kDa molecular weight marker). Thus, eEF-1γ in this peak is probably not alone in interacting with RNA as suggested from earlier studies (Fig. 4A). Further western blot analysis with antibodies to eEF-1β and eEF-1γ confirm that these additional components of the nucleotide exchange complex are present in the same RNA binding fractions as eEF-1γ, suggesting these proteins are part of the eEF-1γ-RNA binding complex (data not shown). Whether or not they are independently capable of binding to RNA remains to be determined.

Western blots with antibody to HAX-1 strongly suggest that the middle peak represents RNA band shift activity due to the binding of HAX-1 (Fig. 8B). Here, there was a good correlation between RNA band shift activity and HAX-1 protein content, with both being the highest in fraction 41. However, band shift activity appears to be a more sensitive technique than western blotting, since considerable RNA binding activity is already present in fraction 39, but HAX-1 protein does not appear on the western blot until fraction 40. Still both methods display peak activity in fraction 41, which supports our conclusion that the middle peak reflects HAX-1 binding.

The protein content of the third peak remains unknown. Since the RNA band shift activity of this peak was quite hetero-dispersed in size, it may reflect the content of multiple mRNA binding proteins, the binding of which are not specific to vimentin’s 3’UTR, i.e., proteins like hRIP or hnRNPs. Clearly, HAX-1 and the eEF-1γβδ containing complexes are separate complexes. At least sufficient protein to be detectable by western blotting with these antibodies was not found in this first peak of RNA band shifting activity.

Calibration of the Superose 6 column with known molecular weight markers indicated that fraction 45 was the position of elution for a 45-kDa protein. Since HAX-1 protein peaks in fraction 41, which corresponds with maximal RNA band shift ability (see arrow), this suggests that HAX-1 interacts with RNA not in a monomeric state but either as a dimer or in complex with other non-identified protein(s). Moreover, neither eEF-1γ nor HAX-1 protein was found in those column fractions that correspond to the size of a monomer, i.e., all migrate ahead of the 45-kDa molecular weight marker. Thus, they must both be components of larger but separable complexes, which are not binding RNA as a single large complex.

**DISCUSSION**

The yeast three-hybrid method has detected proteins that bind to that portion of vimentin’s 3’UTR required for perinuclear localization of a reporter mRNA (22). A variety of experiments presented here demonstrate that these are indeed RNA-binding proteins. First, initial screening methods in yeast confirm that protein binding is RNA dependent. Additional genetic analyses in yeast confirmed that HAX-1 binds specifically only to vimentin’s 3’UTR and not to the IRE or MS2 RNA (Fig. 3). However, it was noted that eEF-1γ displays more flexibility in its RNA binding ability, since some growth also occurred with MS2 RNA. Second, bacterially expressed
hRIP, eEF-1γ and HAX-1 recombinant proteins bind vimentin’s 3′UTR in band shift assays. Moreover, these proteins are within the desired molecular weight range, i.e., ~35–46-kDa, as suggested by UV cross-linking studies (Fig. 2). In the case of HAX-1, binding is dependent on vimentin’s RNA sequence as non-specific RNA, such as BMV RNA, does not effectively compete for binding (Fig. 5). However, two proteins identified here, eEF-1γ and especially hRIP can bind to other RNA sequences in addition to vimentin (Fig. 4). The addition of antibodies to eEF-1γ results in the ablation of binding via in vitro band shift assays further supporting its ability to bind RNA (Fig. 4C). A more detailed study into the requirements of RNA binding for these proteins is underway. Third, a RNA affinity column successfully retrieves both eEF-1γ and HAX-1 from HeLa WCE (Figs 6 and 7). Fourth, fractionation of HeLa cytoplasmic extracts on a sizing column reveals at least three complexes of vimentin 3′UTR-binding proteins (Fig. 8). Here, it appears that the eEF-1γ complex is part of a larger complex of proteins, which can be separated from that complex containing HAX-1. This larger complex may also contain other members of the nucleotide exchange complex. From these experiments we conclude that eEF-1γ and HAX-1, in addition to their known functions, are also RNA binding proteins capable of binding to vimentin’s conserved 3′UTR element. The experiments reported here would suggest that they may be the same 46- and 35-kDa RNA binding proteins revealed by UV-cross-linking studies, however, this has not been rigorously proven.

Additional experiments using the yeast three-hybrid system suggest that the association of HAX-1 with vimentin’s RNA is very strong, since yeast could grow at 50 mM 3-AT whereas IRP-IRE binding can only tolerate 25 mM 3-AT (24). Also, boiling in the presence of SDS was required to remove HAX-1 from the biotinylated RNA SA-PMP complex. This is consistent with our initial attempts to purify vimentin’s 3′UTR binding proteins by conventional RNA affinity columns. Here, we could not remove the RNA binding protein(s) from the affinity column either by washing with increasing salt, 6 M guanidinium hydrochloride, 8 M urea or even distilled water. We had to RNase treat the column material, before we could obtain protein as evidenced by Coomassie stained SDS–polyacrylamide gels. In this case a 35-kDa protein predominated. Because we could not then remove or completely inactivate the RNase, such material was not suitable for subsequent analysis via RNA band shift assays. On the other hand, eEF-1γ appears to not bind so tightly and is dissociated from RNA binding by washing with distilled water. In addition, eEF-1γ can bind to RNA sequences in addition to vimentin’s 3′UTR. Thus, we conclude that HAX-1 binds vimentin’s RNA sequence specifically, but eEF1-γ and especially hRIP are non-specific RNA binding proteins.
To our knowledge this is the first time that either eEF-1γ or HAX-1 have been shown to display RNA binding capabilities. An important question is what does this mean to vimentin mRNA metabolism, and/or perinuclear localization? A review of what is known about these proteins suggests some possibilities.

The eukaryotic elongation factor-1 complex consists of four subunits, eEF-1α, eEF-1β, eEF-1γ and eEF-18. The function of this complex is to induce the efficient transfer of aminoacyl-tRNA to the ribosome, which specifically involves eEF-1α-GTP as the tRNA carrier (equivalent to bacterial EF-Tu). Release of the aminoacyl-tRNA to the ribosome is concomitant with GTP hydrolysis. Interestingly, eEF-1α has been found to be co-localized with β-actin mRNA in cell protrusions and thus may play a role in cell polarity (31). It is the function of the nucleotide exchange complex, eEF-1ββγ, to recycle inactive eEF-1α-GDP to the active eEF-1α-GTP form (32). It has been shown that eEF-1β and -1γ subunits are capable of GDP:GTP exchange activity (equivalent to bacterial EF-Ts) (33). The eEF-1γ subunit is unique to eukaryotes and its function is unknown. It can stimulate, but is not required for, nucleotide exchange activity (34). However, eEF-1γ contains a hydrophobic tail and appears to have an affinity towards membrane and cytoskeletal elements (34). In this capacity it could contribute to the anchoring and translation of mRNAs that are preferentially translated on cytoskeletal- or membrane-bound ribosomes (32). Such translation is unique to eukaryotic cells in agreement with the eukaryotic-specific content of eEF-1γ. Moreover, vimentin mRNA is specifically translated on cytoskeletal-bound ribosomes (35). Our results suggest that eEF-1γ could play an ancillary role in protein synthesis via its ability to bind to RNA. The localization of synthetases, elongation factors and ribosomes into an aggregated structure, which prevents their intermittent diffusion into the cytoplasm, is referred to as the aminoacyl-tRNA channeling hypothesis. In this scenario, the translational efficiency of at least some mRNAs could be enhanced by eEF-1γ’s ability to bind RNA within this structure. Interestingly, eEF-1γ binds BMV RNA in vitro as readily as vimentin’s 3′UTR (Fig. 4) and MS2 RNA in vivo, both of which display stem and loop structures which mimic mRNA structure (23). In this capacity, it would be doubtful that eEF-1γ would have a specific role in perinuclear localization per se, particularly since our preliminary results would indicate that it might recognize overall RNA structure opposed to nucleotide sequence. It is more likely that it would have a general role in translation as related to mRNAs translated in association with the cytoskeleton or in the shut down of overall mRNA translation during mitosis and/or meiosis.

Vimentin gene expression and filament integrity is linked to cell cycle. In eukaryotic cells it has been shown that prior to cell division, the IF network is considerably rearranged to accommodate nuclear membrane disintegration, chromatin condensation and chromosome segregation. Depending on the cell-type, the network either completely disintegrates (BH or MDBK cells) or at the very least collapses around the mitotic spindle (CHO or HeLa) (36–38). In either case, the p34cdc2 kinase phosphorylates the vimentin subunit (on Ser55), which is thought to contribute to the gross rearrangement of the filament network prior to cell division (36). Interestingly, it has been shown that this same kinase phosphorylates both the eEF-1γ and -1δ subunit during meiosis, which affects rates of elongation and protein synthesis (39). However, a similar mechanism in mitosis has not been documented (40). We suggest that it would be counter-productive for the cell to continue synthesizing vimentin at the same time it is disassembling or collapsing the vimentin network. The fact that there is relatively little free vimentin monomer in the cytoplasm, has suggested a co-translational mechanism for vimentin synthesis where dimers and ultimately tetramers are being formed soon after or even during nascent chain synthesis. The head domain (at the amino-terminus) has been shown to be important for filament formation (41). Due to the affinity of eEF-1γ for RNA and more specifically to vimentin’s 3′UTR, it could be playing an additional role in coordinating the translation of mRNAs such as vimentin mRNA with respect to cell cycle. Following cell division, a specific phosphatase removes the phosphate from Ser55 on vimentin and thus vimentin subunits can be re-cycled for IF growth. Perhaps it is this re-polymerization of previous existing subunits which accounts for the requirement of a pre-existing vimentin network to detect vimentin mRNA perinuclear localization in confocal microscopy (22). This would help direct new vimentin synthesis to the site of IFP assembly, which is known to initiate around the nucleus and gradually extend to the cellular periphery to complete filament growth (14–19). Although we have no formal proof of this mechanism, it remains an intriguing suggestion, which awaits further experimentation. At the very least, due to eEF-1γ’s hydrophobic tail, it may contribute to the preferred translation of cytoskeletal- and/or membrane-bound mRNAs such as vimentin, perhaps in coordination with mitosis or meiosis.

HAX-1 was first isolated in a yeast two-hybrid screen using HS1, a substrate of the Src family tyrosine kinases, as bait (29). HS1 expression is limited to hematopoietic and lymphoid cells and thus, it was suggested that HAX-1 might be involved in the regulation of B-cell signal transduction and apoptosis. However, HAX-1 is a ubiquitously expressed protein, so its cellular role is not limited to restricted cell types. Due to an amino acid sequence similarity between cortactin/EMS1 and HS1, cortactin is thought to be the protein form that associates with HAX-1 in non-lymphoid cells (42). More importantly, HAX-1 has a putative hydrophobic transmembrane domain close to its C-terminus (29). Originally the HAX-1 protein was reported to be localized to the outer mitochondrial membrane, and, to a lesser extent, to the endoplasmic reticulum and nuclear envelope (29). But in point of fact these are continuous membranes and HAX-1 distribution along the endoplasmic reticulum and nuclear envelope is evident by confocal microscopy (43). It should be noted that this distribution is similar to what we and others see with in situ hybridization to vimentin mRNA or the GFP:vimentin 3′UTR reporter mRNA (6,22). Due to the location of the HAX-1 protein, it becomes an intriguing candidate for contributing to the perinuclear localization of selected mRNAs. In support of this hypothesis, our data would suggest that binding is specific between HAX-1 and vimentin’s 3′UTR from the experiments in vivo with yeast and since excess non-specific RNA did not compete with vimentin for binding to recombinant, bacterially-expressed HAX-1. In addition, the putative role of HAX-1 as a target for signal transduction events suggests that RNA binding might also be subject to
regulation. Again, this would fit with the presumed rearrangement and pursuant interruption of vimentin mRNA perinuclear localization during nuclear disintegration and cell division. Moreover, a HAX-1 doublet was noted in the western blots of HeLa WCE (Fig. 7) suggesting this protein is a target for post-translational modification, although both species appeared to bind equally to RNA (Fig. 7, lane 2). However, a doublet was not noted in the HeLa cytoplasmic extract used in Figure 8, the reason for which is unknown. Perhaps this reflects a difference in extract preparation, i.e., whole cell versus cytoplasmic extract treated with the detergent NP-40.

In the case of mRNAs localized to the periphery of the cell, it has been proposed that elaborate motors and energy of hydrolysis are required to transport such mRNAs to their final cellular destination. In the case of vimentin, its mRNA need not be specifically transported to a different cytoplasmic location. A simple diffusion, entrapment model would suffice to keep vimentin mRNA concentrated around the nucleus. In this scenario, as vimentin mRNA molecules exit the nuclear pore, they simply diffuse until being trapped by binding to HAX-1 molecules either inserted into the nuclear envelope or along the endoplasmic reticulum. Such a simple mechanism might not require a motor or the expenditure of energy of hydrolysis. However, it has been noted that there are deleterious effects to cell morphology and motility, if the vimentin mRNA is misdirected to the cell periphery (21). Thus, it will be interesting in the future to determine whether or not an active mechanism is required for perinuclear mRNA localization.

The yeast three-hybrid method has been useful in redefining the activity of two previously known proteins, eEF-1γ and HAX-1, as RNA binding proteins. The region of vimentin’s 3′UTR used as ‘bait’ in the three-hybrid method has proved to be both necessary and sufficient for the perinuclear localization of a GFP reporter mRNA. Moreover, localization appears dependent on the presence of the IF network itself, as in SW13 cells lacking any IF network, the reporter mRNA is dispersed throughout the cytoplasm (22). Based on the localization of HAX-1 to the nuclear envelope and endoplasmic reticulum, we propose that it may be actively involved in perinuclear mRNA localization. On the other hand, eEF-1γ is part of a complex responsible for regenerating eEF-1α-GTP as required in protein synthesis. Thus, eEF-1γ may play a more general role in translational regulation with perhaps an additional contribution to the cell cycle control of vimentin synthesis and/or site of translation, i.e., cytoskeletal- or membrane-bound. In summary, the identification of both HAX-1 and eEF-1γ as RNA binding proteins is a novel finding, the importance of which remains to be determined.

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