Purification and Properties of HuD, a Neuronal RNA-binding Protein*

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HuD is a human neuronal specific RNA-binding protein. In this study we have purified HuD and examined its RNA binding properties in detail. HuD binds to mRNAs that contain an AU-rich element with high affinity. In the case of the c-fos AU-rich element, HuD binds to a 27-nucleotide core element comprising AUUUA, AUUUA, and AUUUUUU motifs. Mutation in any two of these motifs abrogates binding. HuD contains two tandem RNA recognition motifs (RRM), a basic domain, and a third RRM. Deletion analysis has shown that only the first and second RRMs are essential for RNA binding. Thus, these specific RNA binding properties support the idea that the HuD regulates gene expression at the post-transcriptional level.

HuD is a human member of the Elav-like neuronal RNA-binding protein family (1). Elav, the founder member of this family, was discovered through genetic studies in Drosophila (2, 3). Mutations in the Elav locus result in a dysfunctional nervous system in which neuroblasts continue to proliferate, fail to differentiate, and migrate inappropriately (4). In addition to its role in development, Elav function is also continuously required for neuronal maintenance. Flies with temperature-sensitive mutations in Elav lose motor control on exposure to the restrictive temperature (5). The human homologues of Elav were independently discovered as the target antigens in paraneoplastic sensory neuropathy (1). This observation suggests that vertebrate Elav-like proteins may also have a critical role in the development and maintenance of vertebrate neurons. Information on the Elav-like family has significantly expanded with the isolation of further homologues from human, mouse, rat and Xenopus (6–9). In Drosophila there appear to be only two genes, elav and rbp9, which differ in their spatial and temporal expression pattern. In higher organisms there appears to be an additional member (Hel-N1 in humans, elrB in Xenopus) that is distinct, in that it is expressed in testis and ovary and in neurons (8, 10). In keeping with their likely role in neurogenesis the vertebrate Elav-like genes are expressed on terminal differentiation of neurons (6, 11, 12).

All Elav-like proteins contain three copies of the RNA recognition motif (RRM), an 80-amino acid domain found in many RNA-binding proteins of diverse function (13, 14). Thus, it is thought that the Elav-like proteins promote neuronal differentiation by post-transcriptional regulation of mRNAs that control cell proliferation and differentiation. This hypothesis has been strengthened by the observation that the Elav-like proteins selectively bind to the 3'-UTR of mRNAs that regulate cell proliferation and differentiation (10, 15). It has been proposed that the Elav-like proteins specifically bind to the short AU-rich segments (AREs or “Shaw-Kamen elements”) that regulate mRNA turnover (16). In particular, studies on Hel-N1 have shown that it specifically selects mRNAs that contain AREs from a total mRNA library (17). Studies on HuD have shown that in crude extracts it specifically binds to the ARE element of c-fos mRNA (18). More detailed analysis of this interaction was not possible, since highly purified proteins were not available. Although many activities have been identified that bind to ARE elements in crude extracts, relatively few have been purified and cloned (19–22). Moreover, after cloning and purification, such activities have not displayed the same specificity displayed in the crude extract (23, 24). In this paper we examine the RNA binding properties of the purified HuD protein and have established that it binds to AREs with high affinity and specificity.

MATERIALS AND METHODS

Construction of a GST-HuD Fusion Protein Plasmid—A cDNA encoding residues 2–373 of HuD was generated using BamHI-linked primers and polymerase chain reaction. The resultant product was digested with BamHI and ligated into BamHI-digested pGEX-T7 (25). Colonies were screened for inserts by Western blotting with monoclonal antibody 16A11 (11). The resultant construct was called pGEX-HuD. The other constructs were generated the same way as described above. The residues of HuD contained in each construct are as follows. For pGEX-HuD I B III, 245–373; for pGEX-HuD III, 279–373. The other constructs were generated the same way as described above. The residues of HuD contained in each construct are as follows. For pGEX-HuD II I B, 2–268; for pGEX-HuD II I, 2–216; for pGEX-HuD I, 28–136; for pGEX-HuD II, 110–216; for pGEX-HuD B, 201–297; for pGEX-HuD B III, 245–373; for pGEX-HuD III, 279–373. The colonies were screened for inserts by SDS-polyacrylamide gel electrophoresis. In the original HuD cloning paper we assigned the initiating AUG codon to nucleic acid residues 95–97 (26). We have now assigned the initiating AUG to residues 116–118 (18). This is in better agreement with HuD cDNAs from other species and the Kozak consensus rule. The numbering system used above reflects this change.

Purification of GST-HuD Protein—Overnight cultures of Escherichia coli BL 21, transformed with each construct, were diluted in 1:50 LB media. At an A600 of 0.4, the cultures were induced with isopropyl-1-thio-D-galactopyranoside (0.1 mM). After 4 h of further growth, cells were spun down and resuspended in 10 ml of buffer A (50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA). The cells were lysed by adding lysozyme and Triton to a final concentration of 0.2 mg/ml and 1%, respectively. The lysates were centrifuged at 12,000 × g for 30 min. The resultant supernatants were loaded onto a glutathione-agarose affinity column (13 mg of protein/ml resin). After washing the column with buffer B (50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1% Triton), each fusion protein was eluted with 50 mM Tris (pH 8.0) and 5 mM glutathione. Active protein was determined either by protein or by RNA-complex formation, pooled, and stored at −70 °C.

Preparation of Labeled RNA Transcripts—Plasmid DNAs were digested without the appropriate restriction enzymes and transcribed in the
presence of \(^2\text{P}\)uridine triphosphate (Amersham Corp.) as described previously (27). pAUFL was linearized with HindIII, yielding a transcript of 251 nucleotides (28). pAU1 was linearized with Ncol, yielding a transcript of 77 nucleotides, pAU2 was linearized with HindIII, yielding a transcript of 115 nucleotides, pAU12 was linearized with HindIII, yielding a transcript of 170 nucleotides. These plasmids were provided by Dr. Ann Bin Shyu. pSP65H-g(\(\gamma\)-globin) was linearized with Sau3AI, yielding 165 nucleotides containing 80 nucleotides of coding sequence and 85 nucleotides of 3'-untranslated region. pSP65H-\(\gamma\) was provided by Dr. Gary Brewer. pARE was generated by subcloning nucleotides 72–190 from pAUFL into pCR1. pARE was linearized with HindIII and transcribed with T7 RNA polymerase to yield a transcript of 248 nucleotides. All transcripts were gel-purified as described previously (27).

Preparation of Ribo-oligonucleotides—Ribo-oligonucleotides were chemically synthesized at a 0.2-\(\mu\)mol scale on a 392 DNA/RNA synthesizer (Applied Biosystems, CA) using RNA phosphoramidites (Glen Research, VA). Ribo-oligonucleotides were end-labeled using T4 kinase and \(\gamma\)-\[^32\text{P}\]ATP to a specific activity of 5 \(\times\) \(10^{6}\) cpm/\(\mu\)mol of oligo and gel-purified.

Gel Retardation Assay—Reaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml bovine serum albumin, labeled mRNA (100 pm), and protein as indicated. Mixtures were incubated at 37°C for 10 min. Following incubation, 4 \(\mu\)l of a dye mixture (50% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol) was added, and 25% of the reaction mixture was immediately loaded on a 1% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The gel was then electrophoresed at 40 V for 2.5 h. The gel was dried on DE81 (Whatman) with a backing of gel-drying paper (Hudson City Paper, NJ) and exposed to XARS film (Eastman Kodak Co.) for 4–5 h at \(-70^\circ\)C.

Nitrocellulose Filter Binding Assay—Reaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml bovine serum albumin, 0.25 mg/ml TRNA, labeled mRNA (100 pm), and purified HuD as indicated. After 10 min of incubation at 37°C, the mixtures were diluted 1:6 with buffer F (20 mM Tris (pH 7.0), 150 mM NaCl, 0.05 mg/ml TRNA) and filtered through nitrocellulose. After washing the filter twice with buffer F, bound radioactivity was determined by Cerenkov counting.

RESULTS

Characterization of HuD-fos mRNA Complex Formation—In previous studies we showed that extracts of E. coli that express HuD support formation of a specific HuD-RNA complex (18). To characterize this interaction further we have made a GST-HuD construct and purified the GST-HuD protein by glutathione affinity chromatography. In the present studies we have used transcripts derived from the 3'-UTR of c-fos mRNA since its ARE element has been functionally defined by mutational analysis (28, 31, 32). The structure of these RNAs and their sequence is shown in Fig. 1. In the majority of the following experiments we use a 214-nucleotide RNA (called AUFL, which is short for AU full-length) that encodes sequence from residue 568 to 781 downstream of the c-fos stop codon. Purified GST-HuD quantitatively converts the AUFL transcript to a protein-RNA complex that migrates slowly on agarose gel electrophoresis. We have previously found that agarose gel electrophoresis provides the best resolution of protein-RNA complexes (33). No complex formation was observed without GST-HuD protein or with purified GST (Fig. 2, lanes 1–4). The amount of complex formed was proportional to the concentration of GST-HuD. Quantitative conversion was observed at 480 \(\text{nM}\) HuD. Complex formation is specific, since no reaction was observed with a control RNA (the 3' end of the human \(\gamma\)-globin mRNA) of similar size and composition that does not contain an ARE (Fig. 3). We have found that complex formation was optimal at 37°C and at pH 7.0. The binding reaction is remarkably resistant to salt (Fig. 4). Moreover, it appears that the complex can be resolved into two components, a slowly migrating species that is salt-sensitive and a fast migrating species that is remarkably stable to salt. We have found that both complexes are readily dissociated by low concentrations (0.005%) of SDS. This sensitivity indicates a noncovalent interaction. The existence of two discrete complexes is also indicated by kinetic studies (Fig. 5). The fast migrating complex forms first and on further incubation is quantitatively converted to the more slowly migrating complex. It is important to note that in this experiment the samples were individually loaded after the indicated time point. Thus, the stated time point is an underestimate of the real incubation time. This is more significant for the early time points.

HuD Binds to c-fos mRNA with High Affinity—The interaction between HuD and c-fos mRNA was further investigated using a nitrocellulose filter binding assay. We employed the same method as originally used for the R17 coat protein (34). A low concentration of labeled RNA (100 pm) was incubated with increasing concentrations of HuD protein as indicated. The reaction mixtures were filtered, and the bound radioactivity was determined. Fig. 6A shows that the formation of the RNA-HuD complex is detectable at 1 \(\text{nM}\), has a midpoint at about 20 \(\text{nM}\), and reaches a plateau above 100 \(\text{nM}\) with about 93% of the input RNA bound. Complex formation with control globin RNA was not detectable under these conditions (Fig. 6A). A plot of the log of complex/free RNA versus the log of HuD concentration reveals a straight line with an intersect on the x axis at 19 \(\text{nM}\) (Fig. 6B). Thus, the binding of HuD to c-fos mRNA is a...
simple molecular reaction with an apparent $K_d$ of 19 nM. In three subsequent determinations, the binding curves were similar, and the $K_d$ values were determined to be 16, 14, and 20 nM, respectively.

HuD Binds to the ARE Segment of c-fos mRNA—We next examined the specificity of binding to the c-fos transcript in more detail. Four transcripts were synthesized (AU1, AU2, AU12, and ARE), which contained the indicated segments of the c-fos mRNA (Fig. 1). Transcript AU12 is a deletion of the ARE. Agarose gel retardation (Fig. 7A) and filter binding assays (Fig. 7B) reveal that HuD binds predominantly to the ARE segment with high affinity ($K_d = 19$ nM). Significantly less binding was observed with AU1 (only 3% binding at 720 nM), AU2 (only 23% binding at 720 nM), and AU12 (only 37% binding at 720 nM). We have further mapped the c-fos binding site using an RNase T1 digestion technique. In this technique the HuD-RNA complex is digested with RNase T1, and the remaining protein-bound fragments are isolated by absorption to nitrocellulose (33). The complete sequence of the AUFL transcript is shown in Fig. 1. RNase T1 digestion yields two major oligonucleotides of 35 and 20 residues and a large number of unresolved species (Fig. 8A, lane 2). The indicated concentrations of HuD or GST were incubated with AUFL transcript. The bound fragments were extracted from the nitrocellulose filter and resolved by polyacrylamide gel electrophoresis. Fig. 8 shows the HuD-dependent retention of three fragments, A, B, and C. A and B appeared to be identical to the 20- and 35-nucleotide T1 digestion markers (lane 2). We then used the 20- and 35-nucleotide T1 fragments as markers to size fragment C. Thus, the extrapolated size of C is 46 nucleotides. To map these fragments in the AUFL sequences, we isolated them from a preparative gel and redigested them with RNase T1. This analysis showed that A corresponded to the 20-nucleotide T1 fragment, that B corresponded to the 35-nucleotide fragment, and that C was composed of the 35-nucleotide fragment (Fig. 8B). This conclusion is independently confirmed by our observation that T1 analysis of the AU12 transcript did not yield any protein-bound fragments (Fig. 9). Thus, we conclude that HuD has high affinity with a segment of AUFL RNA encompassed by the 35-nucleotide T1 fragment (Fig. 1). In some complexes, the G residues adjacent to the 35-nucleotide core T1 fragment
were protected, yielding a 46-nucleotide segment (fragment C). It is difficult to unambiguously assign the protected residue. Protection of the 5' G residue is most consistent with the sizes of the T1 digestion products. At high concentrations of HuD we detect independent but lower affinity binding to the 20-nucleotide fragment (fragment A). Both the 20-nucleotide and 35-nucleotide segment are included in the sequence of AUFL that has been functionally defined as the minimal ARE element (28) responsible for rapid mRNA degradation (Fig. 1).

We next tested whether the 35-nucleotide segment could independently support HuD binding. The 35-nucleotide segment and a series of progressive 3' deletions called 27, 20, and 13 were chemically synthesized (Fig. 10). The 35-nucleotide segment supported complex formation with similar affinity (apparent \(K_d \approx 29\) nM) as the AUFL transcript (Fig. 10). Similar reactivity was also observed with the 27-nucleotide segment (apparent \(K_d \approx 28\) nM), but deletion of a further 7 nucleotides diminished reactivity (only 20% complex formation at 720 nM). Deletion of a further 7 nucleotides yielded no detectable complex formation even at 720 nM. Thus, we concluded that the essential HuD recognition sequences reside in the 27-nucleotide segment. We also synthesized a 23-nucleotide fragment (called 23) that encompasses the minor recognition site identified by the T1 fragment A. As expected, this fragment was bound by HuD, albeit with lower affinity (20% complex formation at 720 nM).

FIG. 10. Deletion analysis of the HuD binding site. The indicated concentration of HuD or GST were incubated with \(^{32}\)P-end-labeled RNAs were incubated with HuD and filtered through nitrocellulose. A, sequences of deletions; B, RNA binding activity. \(\odot\), 27; \(\bullet\), 20; \(\blacktriangle\), 13; \(\Box\), 23.

FIG. 7. A, HuD binds to the c-fos ARE. \(^{32}\)P-labeled RNA (100 pm, 30,000 cpm/pmol UTP) was incubated without protein, with GST, or with the indicated concentration of HuD protein. After 10 min of incubation at 37°C, 25% of the reaction mixtures were resolved on 1% agarose gel. B, HuD affinity with AU1, AU2, and AU12 as determined by nitrocellulose filtration. C, AU1; \(\bullet\), AU2; \(\blacktriangle\), AU12; \(\blacktriangleleft\), ARE; \(\odot\), AUFL; \(\Box\), globin.
establish whether the HuD-fos RNA complex would be displaced by another ARE of similar function but disparate sequence. For this experiment we synthesized the 26-nucleotide core ARE of the Adenovirus IV A2 mRNA. This core sequence is sufficient to destabilize mRNA (21). Fig. 12 shows that addition of the indicated molar excess of the Adenovirus IV A2 core sequence displaced the HuD AUFL complex. No effect was seen with identical molar excess of a control oligonucleotide.

The most discernible structural feature of the 27-nucleotide segment is the arrangement of U residues. We therefore tested the significance of these residues by synthesizing mutant oligonucleotides in which they were changed to G and C residues. The sequences of the mutant oligonucleotides are described in Fig. 13. Substitution of only one of the three U stretches had a modest effect on binding (Fig. 13). The apparent $K_d$ for the 27-nucleotide oligo was 11 nM, whereas the apparent $K_d$ for mutants 27-2, 27-3, and 27-4 was 25, 38, and 62 nM, respectively. Disruption of any two stretches (mutants 27-5, 27-6, 27-7) drastically reduced RNA binding (only 5% at 720 nM HuD). Alteration of all three stretches (mutant 27-8) eliminated binding (no complex formation at 720 nM HuD).

**Analysis of RNA Binding Domains**—The most striking and unique structural feature of the Elav-like family of proteins is the presence and organization of the three putative RNA recognition motifs. In each case the two tandemly arranged RNA recognition motifs are connected to the third RNA recognition motif by a highly basic segment that we have termed the “basic segment.” To determine the domains of HuD required for RNA binding we purified seven mutant proteins that are shown in Fig. 14B. RNA binding affinity was assayed by gel retardation, and protein-RNA complex formation was quantitated by Cerenkov counting. We did not use the nitrocellulose filter binding technique because one of the mutant proteins (HuD I II) did not give similar results on gel retardation and nitrocellulose filter binding assays. All other mutants displayed similar $K_d$ values on using either technique. The apparent $K_d$ values for HuD, HuD I II B, and HuD I II were 16, 98, and 125 nM, respectively. Thus, the third RNA domain is not essential for RNA binding. As expected the third RNA binding domain alone (HuD III) or in conjunction with the basic segment (HuD BIII) displayed very low binding activity (only 5% activity at 7,200 nM HuD). Similarly the basic segment did not bind RNA, nor did it significantly stimulate the activity of HuD I II. HuD I and HuD II are required in tandem, since either alone displayed poor RNA binding (apparent $K_d$ values of 2,100 and 2,000, respectively).

**DISCUSSION**

These results provide new and more extensive information on the RNA binding properties of HuD, a member of the Elav-like family (8). We have shown that HuD specifically binds to the-c-fos ARE. Such ARE elements are conspicuously present in the 3'-UTR of mRNAs that are rapidly and transiently expressed after cellular stimulation (16). Moreover, it is clear that the rapid induction of such mRNAs is the result of an inhibition of ARE-mediated degradation. Thus, the present studies further strengthen the hypothesis that the Elav-like proteins (HuD, HuC, and Hel-N1) control neuronal maintenance and development by the post-transcriptional regulation of gene expression (10, 18). Such rapid and transient expression of genes is of peculiar importance to neuronal cells (37). Neuronal cells are continuously stimulated by neurotransmitters with consequent transient alterations in gene expression. It is not surprising, therefore, that the Elav family has diverged in higher organisms and encompasses three family members. It is important to note that this form of post-transcriptional control occurs in all cell types. It is likely that a homologue of the Elav-like proteins will be expressed in all
cells. Recently such an activity has been cloned and characterized (8, 38).

We have focused on the interaction between HuD and c-fos mRNA because its ARE has been well characterized. When the c-fos ARE was inserted into the 3'-UTR of β-globin mRNA, it decreased the half-life of this stable message from 24 h to 37 min (32). We have shown that HuD binds to the ARE element but not to the adjacent AU-rich elements AU1 and AU2. Although AU1 and AU2 are well conserved in chicken, rat, and human fos mRNA, they appear to contribute little destabilizing activity (32). Using T1 digestion and deleted transcripts we have determined the minimal sequences recognized by HuD. The salient structural feature of the c-fos ARE, as in all AREs, is the presence and organization of AUUUA pentanucleotide sequences (16, 39). The low affinity HuD binding site comprises a tandem AUUUA motif. The major binding site, which exhibits a similar affinity as the entire transcript, is comprised of an AUUUA element, an AUUUA hexamer, and an AUUUAUA heptamer (Fig. 8). The low affinity of the tandem AUUUA and mutation of the third AUUUA does not significantly affect binding, indicating that the AUUUA motifs themselves are not essential for binding. Disruption of the AUUUAUA heptamer, however, in addition to mutation of the AUUUA or AUUUAUA motifs abrogates binding. From these results we conclude that HuD does not recognize a primary sequence but binds to a structure that requires the participation of at least two of the three U stretches. This idea fits with our observation that HuD binds to and is displaced by other AREs of disparate sequence but presumably similar structure.

Several ARE binding activities have been previously described (19–22, 40–42). The majority of these studies have utilized a UV cross-linking assay. Thus it is difficult to make a comparison with our results. One of these activities (AUF1) has been purified and cloned and is clearly a different gene product (24). In contrast to the tripartite structure of HuD, AUF1 contains two RRMs and a C-terminal glutamine-rich domain. The RNA binding properties shown here most resemble the tripartite structure of HuD, but presumably similar structure.

Fig. 14. A, analysis of RNA binding domains. The indicated concentrations of purified HuD derivatives were mixed with 32P-labeled AUFL RNA (100 pM, 30,000 cpm/pmol UTP). Following incubation at 37°C for 10 min, 25% of the reaction mixtures were resolved on 1% agarose gel. B, structure of the mutant HuD derivatives. The residues of HuD contained in each construct are as follows: for pGEX-HuD I II B, 2–268; for pGEX-HuD I II, 2–216; for pGEX-HuD I, 28–136; for pGEX-HuD II, 110–216; for pGEX-HuD I II B III, 2–216; for pGEX-HuD II III, 245–373; for pGEX-HuD III, 279–373. C, quantitative analysis of mutant binding activity. ▲, HuD; ○, I II B; ●, I II; △, I; ●, II; □, B; ○, III; △, III.
correct, purified HuD may provide a reagent to assay and characterize such an activity and thereby reconstitute the first steps in selective degradation.

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