RNA binding specificity of a *Drosophila* snRNP protein that shares sequence homology with mammalian U1-A and U2-B'' proteins

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ABSTRACT

We have characterized a recombinant *Drosophila melanogaster* RNA binding protein, D25, by virtue of its antigenic relationship to mammalian U1 and U2 small nuclear ribonucleoprotein (U snRNP) proteins. Sequence analysis revealed that D25 bears strong similarity to both the human U1 snRNP-A (U1-A) and U2 snRNP-B'' (U2-B'') proteins. However, at residues known to be critical for the RNA binding specificities of U1-A and U2-B'' D25 sequence is more similar to U2-B''. Using direct RNA binding assays D25 selected U1 RNA from either HeLa or *Drosophila* Kc cell total RNA. Furthermore, D25 bound U1 RNA when transfected into mammalian cells. Thus, D25 appears to be a *Drosophila* homolog of the mammalian U1-A protein, despite its sequence similarity to U2-B''.

INTRODUCTION

It is now well established that an RNA recognition motif (RRM) of 80 amino acids forms the core of the RNA binding domain of a number of important cell proteins (1). The RRM is present in a large family of proteins, most of which are involved in some aspect of RNA processing, and is characterized by the presence of two especially conserved sequence elements, RNP-1 and RNP-2 (2). The structure of the U1 snRNP-A (U1-A) RNA binding domain, which encompasses its amino-terminal RRM, has been determined both by X-ray crystallography (3) and two-dimensional nuclear magnetic resonance spectroscopy (NMR) (4). The derived structure contains a four-stranded anti-parallel beta sheet predicted to be common to all RRM-containing proteins. RNP-1 and RNP-2 exist within adjacent beta strands within this beta sheet, and are believed to provide surfaces for generalized RNA binding (beta strands 1 and 3), while less highly conserved loop structures adjacent to the beta sheet are thought to contribute surfaces important for determining RNA binding specificity (1).

Two of the best characterized RRM-containing RNA binding proteins are the human U1-A and the U2 snRNP-B'' (U2-B'') proteins (5–9). U1-A and U2-B'' each possess two RRMs; their N-terminal RRMs (RRM 1) are 77% identical (10,11) and have

been shown to be necessary for binding to their cognate RNAs. U1-A recognizes loop 2 of U1 RNA (6,12) and U2-B'' recognizes loop 4 of U2 RNA in the presence of the U2 snRNP-A' (U2-A') protein (5,7). The RNA binding sites for U1-A and U2-B'' on U1 RNA and U2 RNA, respectively, are similar in nucleotide sequence. Mutational analyses have demonstrated that amino acid residues immediately N-terminal to the RNP-1 sequence of RRM 1 are important determinants of RNA binding specificity for both U1-A and U2-B'' (5,7,8). However, these experiments suggest that other specificity determinants may exist within their RNA binding domains.

One way to gain a better understanding of the RNA binding specificity of human U1-A and U2-B'' is to isolate homologs from other organisms and analyze regions of sequence conservation. The high degree of conservation between the human and *Drosophila* U1 and U2 RNAs in the binding sites for U1-A and U2-B'' (13), the availability of *Drosophila* tissue culture cells (Kc cells), the proposed simplicity of *Drosophila* Kc cell snRNP protein composition (14,15), and the potential for genetic studies (16) makes *Drosophila* a useful system for such analyses.

We have cloned and characterized a cDNA encoding a 25 kD protein, D25, from a *Drosophila* expression library. D25 possessed sequence elements characteristic of both human U1-A and U2-B''. However, at sequences previously identified as critical for their differing RNA binding specificities (5,6), D25 more closely resembled U2-B''. In direct RNA binding assays D25 bound *in vitro* to naked U1 RNA from both HeLa and Kc cells and associated with U1 snRNPs when expressed in mammalian cells, in a manner similar to the U1-A protein. Thus, D25 appears to be a *Drosophila* counterpart to the mammalian U1-A protein, despite its similarity to U2-B'' at residues involved in determining RNA binding specificity.

MATERIALS AND METHODS

Enzymes, radionucleotides, vectors and host strains

Enzymes were obtained from United States Biochemicals, Boehringer Mannheim, Promega Biotech, or New England Biolabs. Radionucleotides were obtained from ICN or

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Figure 1. Comparison of D25 translation product with *Drosophila* Kc cell proteins using a human anti-(U1/U2) RNP serum. Transcripts encoding D25 were translated *in vitro* in the presence of ^{35}S methionine and electrophoresed on a 12% acrylamide gel (lane 1) adjacent to 100 μg of a Kc cell S100 fraction, followed by electroblotting onto nitrocellulose. The lane containing the Kc cell proteins was probed with the same human anti-(U1/U2) RNP serum used to isolate the D25 clone, followed by probing with ^{125}I conjugated protein A (lane 2). Protein molecular weight markers, in kD, are indicated.

Amersham. The pGEM vector and the NM522 *E. coli* host strain were purchased from Promega Biotech. The pET vector and the *E. coli* host strain BL21(DE3)pLysS were the generous gifts of Dr. William Studier (Brookhaven National Laboratory).

Library screening, cDNA cloning and DNA sequence analysis

Isolation of cDNA clones encoding U1-A and U2-B' proteins have been described (5,12). cDNA clones encoding D25 were obtained by screening a *Drosophila* embryonic cDNA expression library (provided by Dr. Tao Hsieh, Duke University) according to published procedures (17) using a human autoimmune serum with reactivities against U1 and U2 snRNP specific proteins (18). Full length cDNA clones were isolated by nucleic acid hybridization screening according to standard techniques (19). DNA sequence analysis was performed using Sequenase Version 2.0 T7 DNA polymerase (USB).

In vitro transcription and translation

The D25 cDNA was subcloned downstream of a T7 polymerase promoter in the pGEM-3Zf transcriptional vector, transcribed, and translated in rabbit reticulocyte lysate as recommended by the supplier (Promega Biotech). Clones of human genomic U1, U2, and U6 RNAs were gifts from Nouria Hernandez (Cold Spring Harbor Laboratory), Alan Weiner (Yale Univ.), and Ram Reddy (Baylor College of Medicine), respectively. U1DSL1 (U1 RNA deleted for stem-loop 1) and U1DSL2 (U1 deleted for stem-loop 2) clones were constructed as previously described (20). These genes were subcloned into pGEM transcriptional vectors, linearized by the appropriate restriction enzyme, and transcribed *in vitro*.

Western blot analysis

Kc cells were grown in D22 media (GIBCO-BRL) and cytoplasmic S100 fractions were prepared as described (21). 100 μg of protein were loaded per lane on a 12% SDS-acrylamide gel and electrophoresed. The separated proteins were

Figure 2. Comparison of the complete deduced D25 amino acid sequence with those of the human U1-A and U2-B' proteins. Identical residues are indicated by vertical lines. Dashed lines indicate the lack of corresponding amino acids in either D25 or U2-B' with those present in U1-A. The numbering corresponds to the U1-A amino acid sequence. The RRM2s extend from the RNP-2 sequences; the amino-terminal RRM2s (RRM 1) all terminate at the conserved SER residue at position 91 in the U1-A protein, while the C-terminal RRM2s (RRM 2) terminate at the end of the protein. Unshaded boxes represent the highly conserved RNP-1 and RNP-2 sequences, while the shaded box represents the pentamer sequence involved in RNA binding specificity (2; see text). The black bar is below the U2-B' sequence shown to be involved in RNA binding specificity and the U2-A' interaction in the studies of Scherly et al. (7,8), while asterisks above the sequence denote other residues in U2-B' also shown to be involved in U2-A' interactions.

electroblotted onto nitrocellulose, and the filter was incubated with a human anti-(U1/U2) RNP serum, followed by incubation with ^{125}I conjugated protein A (ICN).

Electrophoretic mobility shift analysis

^{35}S labeled D25 translation product was incubated with various *in vitro* transcribed RNAs in the following buffer (KNET-3): 50mM Tris (pH 7.4), 150 mM NaCl, 0.05% Nonidet P-40, 1mM DTT, 10mM MgCl_2 , 2% PVA, 0.1 mg BSA per ml, 0.5 mg *E. coli* tRNA per ml, 0.125 mg polyA per ml, 40 mM vanadyl ribonucleotide complexes (VRCs), and 50 units of RNasin per ml. Following incubation at 37 degrees for twenty minutes the samples were electrophoresed on a native 5% acrylamide-90mM Tris-borate (pH 8.3) gel at 4 degrees.

Overexpression of protein in *E. coli*

The cDNA encoding D25 was subcloned into the pET 3C vector (22) in the correct reading frame behind sequences encoding the

Figure 3. Mobility shift analysis of RNA-protein complexes involving D25. D25 was transcribed and translated *in vitro*, and incubated with one ug of various *in vitro* transcribed human RNA species (or total HeLa cell RNA) as described in Materials and Methods. The RNA species used is noted above each lane; tRNA is present as a competitor in each sample. The U1DSL1 and U1DSL2 constructs lack stem loop 1 and 2, respectively, as described in the Materials and Methods. The position of free (F) and bound (B) protein is indicated.

first eleven amino acids of phage T7 gene 10 protein (g10), producing the clone g10-D25. g10-A and g10-B" clones were constructed as previously described (5,20). These constructs were transfected into *E. coli* strain BL21(DE3)pLysS, resulting transformants induced for expression, and bacterial protein extracts prepared as previously described (5).

RNA binding and immunoprecipitations

Four mg of Protein A Sepharose beads (Sigma) were incubated with antiserum directed against the g10 epitope tag (Dan Kenan, unpublished data), and washed thoroughly with NET 2 buffer (50 mM Tris pH 7.4, 150mM NaCl, 0.05% Nonidet P-40). Bacterial protein extracts containing either g10-D25, g10-A, or g10-B" were incubated on ice with the bead-antibody complex and then washed with NET 2 buffer. The resulting bead-antibody-g10-protein complex was then incubated with 32 P-labeled HeLa or Kc cell whole cell extract (WCE) or deproteinized RNA. Incubation was carried out at 37 degrees for 5 minutes in KNET-3 buffer followed by extensive washing, phenol-chloroform extraction, ethanol precipitation and electrophoretic analysis of co-immunoprecipitated RNAs on 5% acrylamide, 8.3M urea gels.

COS cell transfections

An *Xba*I-*Eco*RI insert from the g10-D25 clone, containing the entire D25 coding region fused to the g10 epitope tag, was subcloned into a pBC derived mammalian expression vector (22,23). This construct was transiently transfected into COS cells, followed by 32 P labelling of the cells with orthophosphate and whole cell extract preparation. As a control, vector alone was also used for transfection and extract preparation. Anti-g10 serum was used to immunoprecipitate g10-D25 from the whole cell extract, and co-immunoprecipitated RNAs were analyzed as described above. Details of the COS cell transfection will be described elsewhere (24).

Figure 4. Co-immunoprecipitation of U1 RNA with g10-D25. Equivalent volumes of g10-D25 (lanes 2 and 3) or g10 only (lanes 1 and 4) *E. coli* protein extracts were incubated under RNA binding conditions (See Materials and Methods) with total RNA from 32 P labeled HeLa (lanes 1 and 2) or Kc cells (lanes 3 and 4). Anti-g10 serum was used to co-immunoprecipitate bound RNAs which were analyzed on an 8% denaturing polyacrylamide gel. The location of U1 and U2 RNAs in the HeLa total lane (M) are noted.

RESULTS

Drosophila protein D25 shares sequence similarity with both the mammalian U1-A and U2-B" proteins

A *Drosophila* embryonic cDNA expression library was screened using a human autoimmune serum that contained antibodies against both human U1-A and U2-B", as well as a number of other U1 and U2 snRNP proteins and U1 RNA. This screen yielded a partial clone that was used to isolate a full length clone by nucleic acid hybridization screening of the same library. A number of isolates were analyzed; each contained some portion of the same insert. A full length insert was subcloned into a transcriptional vector and completely sequenced. The insert was 806 nucleotides and contained an open reading frame that encoded a protein with an estimated molecular weight of 25 kD that was termed D25. Northern blot analysis of Kc cell poly (A)+ RNA probed with antisense D25 transcript demonstrated the presence of a transcript similar in size to the D25 cDNA clone (data not shown). The size of the encoded protein was verified by gel electrophoresis of *in vitro* translated D25 (Figure 1, lane 1). The antiserum that was used for immunological screening of the library was then used to probe a Western blot of *Drosophila* Kc cell protein. A protein of 25 kD was detected (Figure 1, lane 2) that was shown to comigrate with the D25 *in vitro* translation product. A major band was also detected at approximately 32 kD (Figure 1, lane 2). Since the serum used to probe the Western blot contained antibodies against a number of proteins, it was not unexpected that multiple bands would be detected. However, we cannot rule out the possibility that the 32 kD protein is immunologically related to D25. Our inability to isolate the clone expressing the 32 kD protein could be due to a low abundance of the corresponding mRNA.

Analysis of the deduced D25 protein sequence revealed the presence of two RRM's similar to those of the human U1-A and U2-B" proteins (Figure 2). D25 has 77 percent identity to U1-A and 72 percent identity to U2-B" within their colinear residues.

Within RRM 1 D25 has 82 percent identity to U1-A and 77 percent identity to U2-B", and D25 has 83 percent identity to both U1-A and U2-B" in RRM 2. Alignment of the protein sequences demonstrates that D25 contains regions of similarity to either U1-A or U2-B" where those two proteins differ from one another. Most significantly, D25 is identical to U2-B" in a five amino acid sequence (pentamer sequence; see Figure 2, shaded box) demonstrated by Bentley and Keene (2) to confer U2 RNA recognition when substituted into the corresponding position of U1-A and might thus be expected to bind U2 RNA. The molecular weight of D25 is also more similar to U2-B" than to U1-A.

D25 binds U1 RNA *in vitro*, in a manner similar to the mammalian U1-A protein

In order to determine the RNA binding specificity of D25, RNA binding assays were performed. Initially, *in vitro* translated, ³⁵S labeled D25 and *in vitro* transcribed RNAs were used in a mobility shift assay. D25 was incubated with either total HeLa RNA, U2 RNA, U6 RNA, full length U1 RNA or U1 RNA deleted for stem-loop I (U1DSL1) or stem-loop II (U1DSL2) and then subjected to electrophoresis under non-denaturing conditions. A shift in the migration of D25, indicating complex formation, occurred in the presence of total HeLa RNA, full length U1 RNA or U1DSL1, but not in the presence of U1DSL2 (Figure 3). These findings demonstrate that D25 binds to U1 RNA under these conditions and suggests that it binds to stem-loop two, which

Figure 5. Incorporation of g10-D25 (lane 1), g10-A (lane 2), g10-B" (lane 3), and g10 only (lane 4) into snRNPs using Kc whole cell extracts. Equivalent volumes of the bacterial protein extracts were incubated with Kc WCEs and processed as described in Materials and Methods. M denotes Kc cell total RNA; lane 5 is RNA co-immunoprecipitated using an anti-(U1/U2) RNP serum. The position of U1 and U2 RNAs are indicated.

is the known binding site for the human U1-A protein (6,12). These results were in contrast to expectations based on amino acid sequence comparison of the D25, U1-A and U2-B" proteins. We therefore employed an immunoprecipitation assay as a confirmatory method to determine the RNA binding specificity of D25. The coding region of D25 was fused in the correct reading frame with the first eleven amino acids of the phage gene 10 leader peptide (g10-D25). The fusion protein was overexpressed in *E. coli* and a protein extract was prepared. The g10-D25 *E. coli* extract was incubated with ³²P-labeled HeLa cell or Kc cell RNA. As a control, extract was prepared from cells that had been transformed with the expression vector alone (g10 extract). The resulting complexes were immunoprecipitated using anti-g10 serum and co-precipitated RNAs were analyzed. Figure 4 shows that while no RNA co-immunoprecipitated with control extracts (lanes 1 and 4), g10-D25 bound to U1 RNA from

Figure 6. Co-immunoprecipitation of ³²P labelled COS cell RNA following transfection with g10-D25. The eukaryotic expression vector pBC (23), containing the g10-D25 insert, was transfected into COS cells. Following transfection, COS cell RNA was ³²P labeled and g10-D25-associated RNAs were identified by anti-g10 immunoprecipitation. Lane M is total COS cell RNA; lane 1 is an anti-g10 immunoprecipitation of COS cells transfected with control vector containing no insert and lane 2 is an anti-g10 immunoprecipitation of COS cells transfected with the g10-D25 construct. Lane 3 is an anti-(U1/U2) RNP immunoprecipitation of total COS cell RNA. The positions of U1 and U2 RNAs are indicated.

Figure 7. Comparison of the amino acid sequences of D25, U1-A and U2-B" relative to the proposed tertiary structure of the U1-A protein (according to the alignment used in ref. 1). Emphasis is placed on the similarities and differences within various structural regions, as defined by the crystal and NMR structures of the U1-A protein (3,4). The first residue of beta strand 1 corresponds to the beginning of the RRM; RRM 1 terminates at U1-A residue 91, while RRM 2 terminates at the end of the protein. Sequences between the two RRM s that are dissimilar between the three proteins have been omitted.

both HeLa and Kc cells (lanes 2 and 3) in this assay, in agreement with the data from mobility shift experiments (Figure 3).

D25-RNA interactions in whole cell extracts

We assayed the ability of g10-D25, g10-A and g10-B" to associate with U snRNPs in whole cell extracts (WCEs) from Kc cells. The experiments were performed as described above for the RNA binding assays, except that WCEs were substituted for deproteinized RNA. This assay presumably tests the ability of the recombinant proteins to exchange with their *in vivo* counterparts, although we cannot be certain that the proteins are not interacting with a small amount of free RNA. For comparison, an anti-(U1/U2) RNP serum was used to co-immunoprecipitate both U1 and U2 RNA (lane 5). The results (Figure 5) show that U1 RNA and a lesser amount of U2 RNA co-immunoprecipitated with g10-D25 (lane 1) and g10-A (lane 2), while only a minor amount of U2 RNA co-immunoprecipitated with g10-B" (lane 3). Thus, D25 and the U1-A protein exhibited identical incorporation patterns in this experiment.

D25-RNA interactions resemble those of U1A-RNA in transfected mammalian cells

To determine whether D25 could associate with mammalian U snRNPs *in vivo*, the g10-D25 coding sequence was subcloned into a mammalian expression vector and transfected into COS cells as described. Immunoprecipitation of ³²P-labelled WCEs from the g10-D25 transfected cells using anti-g10 serum showed that *in vivo* produced g10-D25 bound to U1 RNA (Figure 6, lane 2), even in the presence of endogenous U1-A protein. Under these conditions a minor amount of U2 snRNA was detected; this may reflect an interaction between U1 and U2 snRNPs in the extract, as similar results are obtained when using g10-A constructs for transfection (24). A minor amount of background binding to U1 RNA was detected with the vector control (lane 1); however, the relative intensities of the U1 RNA bands in lanes 1 and 2 clearly indicate that U1 RNA was specifically co-immunoprecipitated from cells transfected with g10-D25. Thus, when transfected into mammalian cells, D25 behaved similarly to the human U1-A protein (24).

DISCUSSION

We have characterized a novel cDNA from *Drosophila* that encodes a 25 kD protein, referred to as D25. An mRNA of approximately 0.85 kb was detected in *Drosophila* Kc cell poly (A)+ RNA representing D25. In addition, a Kc cell protein that was recognized by the antiserum initially used to isolate the D25 cDNA clone was shown to comigrate with D25 translation product (Figure 1). These data show that D25 is expressed in Kc cells. D25 was found to bind both mammalian and Kc cell U1 RNA *in vitro*, in a similar manner to the human U1-A protein. Human U2-B" associated only with U2 RNA from Kc whole cell extracts, demonstrating a direct interaction with U2 snRNPs. The minor amounts of U2 RNA (relative to U1 RNA) co-immunoprecipitated with D25 and U1-A likely represent complexes containing both U1 and U2 snRNPs that formed in the extract. Thus, these complexes probably do not involve direct interactions between U2 RNA and U1-A or D25, but rather a U1 snRNP-U2 snRNP interaction. In any case, D25 and U1-A show identical patterns of RNA binding in these experiments, suggesting that D25 is a *Drosophila* counterpart to the mammalian U1-A protein. D25 binding to U1 RNA did not require the

presence of the U1-70K protein, as has been reported in one instance for the human U1-A protein (25).

These results were unexpected because D25 was found to possess identity with U2-B" at residues previously shown to be important for RNA binding specificity (Figure 2; ref. 5,6). Recent reports have implicated amino acid residues in β -strand two and the beginning of loop 3 of the first RRM of the U1-A and U2-B" proteins as being critical for their differing RNA binding specificities (5–8; compare Figures 2 and 7). A five amino acid pentamer sequence from U2-B" (see Figure 2, shaded box) has been shown to confer U2 RNA binding *in vitro* when substituted into the corresponding position in U1-A (construct A/B".1A; ref. 5). The A/B".1A chimeric construct retains the ability to bind U1 RNA. A/B".1A is identical to D25 from the first residue of RRM 1 to the end of RNP-1, including the pentamer sequence, except for a single amino acid (GLU21; Figure 2). On the basis of these findings D25 might be expected to bind to naked U2 RNA, as well as U1 RNA *in vitro*. However, the results of electrophoretic mobility shift and immunoprecipitation experiments demonstrated that, *in vitro*, D25 binds solely to U1 RNA from both HeLa and Kc cells. While we have not detected a specific interaction of D25 with U2 RNA from *Drosophila*, we cannot rule out the possibility of an indirect interaction *in vivo* involving protein-protein interactions.

Based on the data presented above, a sequence comparison of the RNA binding domains of U1-A, U2-B", and D25 may provide further information on residues involved in determining the RNA binding specificity of these proteins. Several conservative changes can be seen between D25 and A/B".1A in α -helix 2 of RRM 1 (compare D25 with A, Figure 7), but this region of the protein is predicted to project away from the presumptive RNA binding face (1). The only other differences between the two proteins within RRM 1 exist in β -strand four which lies on the putative RNA binding face and which may also be involved in RNA recognition. Three nonconservative changes (83ARG-GLN, 85GLN-ALA, 87ALA-SER) reside within a five amino acid stretch in β -strand four, where U1-A, U2-B" and A/B".1A are all identical (Figure 7). It is possible that β -strand four of RRM 1 of D25 may be involved in discriminating against U2 RNA binding. This type of discrimination may explain why we were unable to detect D25 binding to U2 RNA, even though it possesses the pentamer sequence, and why exchange of the U2-B" pentamer for the corresponding U1-A sequence allows binding of the A/B".1A to U2 RNA. However, it is also possible that residues outside of the RRM contribute to the overall RNA binding specificity (9).

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