Published online June 4, 2004

3070–3082 *Nucleic Acids Research, 2004, Vol. 32, No. 10*
DOI: 10.1093/nar/gkh627

The *Drosophila* Bruno paralogue Bru-3 specifically binds the EDEN translational repression element

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Received March 9, 2004; Revised April 26, 2004; Accepted May 10, 2004

ABSTRACT

We reported in our previous work that the EDEN-dependent translational repression of maternal mRNAs was conserved between *Drosophila* and *Xenopus*. In *Xenopus*, this repression is achieved through the binding of EDEN to the Bruno-like factor, EDEN-BP. We show in the present work that the *Drosophila* Bruno paralogue, the 45 kDa Bru-3 protein (p45), binds specifically to the EDEN element and acts as a homodimer. We describe for the first time a previously undetected 67 amino acid domain, found in the divergent linker region, the Ism domain (Ism stands for linker-specific motif). We propose that the presence of this domain in a subset of the Bruno-like proteins, including Bru-3, EDEN-BP and CUG-BP but not Bruno nor its other paralogue Bru-2, might be responsible for specific RNA recognition. Interestingly, comparative structural analyses using threaders and molecular modelling suggest that the new domain might be distantly related to the first RNA recognition motif of the *Drosophila* sex-lethal protein (sxl). The phylogenetic analyses and the experimental data based on its specific binding to the EDEN element support the conclusion that Bru-3 is an EDEN-BP/CUG-BP orthologue.

INTRODUCTION

Post-transcriptional control has been recognized as essential in the numerous and complex steps which lead from gene transcription to the production of an active protein. Oocytes and embryos have proved to be powerful tools for probing many of its aspects, such as mRNA stability and translational control (1,2). One reason is transcriptional silencing of the oocyte nucleus involved in the lengthy and complex meiotic events and of the embryo nuclei because of their high rate of replication cycling, preventing the occurrence of any significant transcription. Resumption of massive transcription does not occur before the mid-blastula transition (called the blastoderm stage in *Drosophila*). Therefore, before this stage, the on/off switch of gene expression relies essentially on control of maternal mRNAs (1). Indeed, mRNA contains a combination of *cis* sequences that encode appropriate addressing and use during cell differentiation and/or development. These properties may be encoded throughout the mRNA length, although the 3′-UTR is an essential repository for *cis* regulatory sequences. Not only is this RNA code necessary but other factors are required to translate the encoded programme into function. RNA binding proteins, an extraordinarily complex class of proteins, are devoted to this task (3–5). Given the complexity and diversity of RNA binding proteins, they have been classified into subgroups, very often on the basis of their sequence features. Indeed, binding of these proteins to their target sequences is achieved through characteristic RNA binding domains in combination with more divergent regions that provide the specificity either *per se* or by interacting with other proteins (4).

Within this diverse group of proteins, one of the best characterized RNA binding domains is the highly conserved RNA recognition motif (RRM), sometimes referred to as the RNP or RBD (6). The presence of RRMs in a protein does not provide information on its biological function (for example whether it is a splicing factor or a translational repressor), neither does it provide clues as to its target sequences. It only shows that the protein is likely to bind RNA. Among hundreds of RRM-containing proteins, proteins similar to the *Drosophila* translational repressor Bruno have been classified as Bruno-like proteins on the basis of phylogenetic analysis (7). They are also called CUG-BP, etr-3 like factors or CELF proteins (8). EDEN-BP, first identified as a translational repressor in *Xenopus* eggs, is 90% identical to the human CUG-BP, therefore it is considered to be a member of the CELF proteins (9). All these proteins contain three copies of

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the highly conserved 80–90 amino acid RRM. Two of them are localized in the N-terminal part of the protein and are separated by a divergent linker region from the most conserved C-terminal RRM (10). Despite their high conservation, these proteins are involved in different biological functions. Sometimes, the same protein fulfils different roles. For example, the human CUG-BP, considered a player in Steinert myotonic dystrophy disease (DM1), was first shown to act as a splicing regulatory factor (11). Its role in translational regulation is now well documented (12). As a matter of fact, CUG-BP is a multifunctional protein which shuttles between the nucleus and the cytoplasm, acting both as a splicing factor and a translational repressor or activator, depending on its location and its target RNAs (13). The only function reported so far for EDEN-BP is as a translational repressor through deadenylation (9). However, given its 90% identity with CUG-BP, one can expect it to be orthologous to CUG-BP and have similar properties and, indeed, CUG-BP acts as a deadenylation factor (14).

Bruno is also a translational repressor, shown to be essential for various aspects of pattern formation in the *Drosophila* oocyte (15–17). Nevertheless, unlike CUG-BP, it is found only in the cytoplasm and it is specific to germ cells. The best analysed form is the oocyte-specific 67 kDa protein, the 83 kDa testis-specific form being poorly studied, if at all (16).

What is the molecular basis for these variations in localization and function? All these proteins have linker and auxillary regions which are very divergent. Those domains whose structure and function have been poorly investigated may account for their binding specificity and functional diversity. As an example, Bruno has a long N-terminal peptide unrelated to any domain of the CELF proteins reported so far (16). Bruno may have acquired specialized functions in germ cells thanks to this domain.

In the course of our search for the *Drosophila* EDEN-dependent translational repressor, Bruno was first considered as the likely candidate. Sequencing of the whole *Drosophila* genome and the cDNA sequencing programme carried out by the BDGP consortium led to the identification of Bru-2 and Bru-3, two Bruno paralogues.

We show in this work, using a phylogenetic and experimental approach, that Bru-3 is more related in many aspects to EDEN-BP and CUG-BP than Bruno, despite the presence of only one N-terminal RRM in Bru-3. A careful analysis of the divergent linker region reveals a small linker-specific motif (that we called lsm), found only in Bru-3, EDEN-BP, CUG-BP and their orthologues. Interestingly, threading programs allowing sequence–structure comparisons suggested that the Bru-3 lsm is included in a 67 amino acid domain that might adopt a fold related to that of the RRM domains (18). Using UV cross-linking experiments and gel shift assays, we showed that the 45 kDa Bru-3 (p45) specifically binds a synthetic EDEN $(UGUA)_{12}$ and a $(UG)_{15}$ repeat element and acts as a homodimer. Moreover, in endogenous protein extracts from *Drosophila* ovaries and embryos, only p45 was shown to bind EDEN. Neither the 67 kDa Bruno nor the 75 kDa Bru-2 showed any binding under the same experimental conditions.

Our data are consistent with Bru-3 being a *Drosophila* EDEN-BP/CUG-BP orthologue.

MATERIALS AND METHODS

Bioinformatic analysis

The EDEN-BP amino acid sequence was used as a query in a tblastn search of the *Drosophila* genome sequence database (http://www.flybase.org). The identity and similarity percentages were calculated using PSI-BLAST (19).

The MEME program (http://meme.sdsc.edu/meme/website/meme.html) was used to compare and identify the most conserved regions of the Bruno-like proteins. The identified domains were manually aligned with Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) using the PAM250 matrix. Phylogenetic analyses were performed using neighbor-joining distance methods on the CLUSTAL X sequence alignment (20). Bootstrap N-J commands (N = 1000) of CLUSTAL X were used to generate a bootstrapped tree (21,22). The tree thus generated was visualized with Tree View 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Sequence threading onto three dimensional (3D) structures was carried out with the meta-server (http://bioserv.cbs.cnrs.fr) (23). Potentially compatible folds were further evaluated automatically through partial and then complete modelling using TITO (tool for incremental threading optimization), SCWRL and MODELLER 6.2 (24–26). Modelled structures were evaluated using the Verify3D and PROSA programs (27,28).

RNA-expressing vector and *in vitro* transcription

To generate a $(UGUA)_{12}$-expressing plasmid, the artificial EDEN $(TGTA)_{12}$-containing sequence was cut out from the C06 construct by XbaI and SalI digestion (29). After purification in low melting point agarose gel, the DNA fragment was cloned into the XbaI and SalI sites of plasmid pBluescript SK. Non-specific sequences between the vector T7 promotor and EDEN were eliminated with SpeI and KpnI digestions. To generate a $(UG)_{15}$-expressing plasmid, the primers 5′-ctag$(tg)_{15}$a and 5′-gatct$(ca)_{15}$ were hybridized to generate a double-stranded oligonucleotide, with SpeI and BglII flanking sites used in subcloning into the Bluescript SK vector.

The ^{32}P-labelled RNA probes were obtained by *in vitro* transcription with a Promega Riboprobe kit using [α-^{32}P]UTP as a radioactive precursor. EDEN sense $(UGUA)_{12}$ was transcribed by T7 RNA polymerase after linearization of the vector by BglII digestion. For the EDEN antisense $(UACA)_{12}$ probe the plasmid was digested with SpeI and transcribed by T3 RNA polymerase. The $(UG)_{15}$ probe was obtained by transcription with T3 RNA polymerase after plasmid linearization by BglII digestion.

Protein expression in bacteria and S2 cells

Cloning procedures. The bacterial plasmid vectors expressing full-length Bru-3 and His-tagged truncated Bru-3 proteins were constructed by cloning PCR-amplified regions of Bru-3 cDNA into the pTrcHis-TOPO-expressing plasmid according to the manufacturer's recommendations (Invitrogen).

The amplification step was carried out with a high fidelity Pfu polymerase (Promega) in a MJ Research minicycler according to the following protocol. A 2 min initial denaturation step at 94°C was followed by 30 cycles of

denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 74°C for 1 min. After a final elongation at 74°C for 10 min with Pfu, a Taq polymerase step was added as cloning of the resulting fragments in the TOPO vector required the presence of a protruding adenosine residue. This was done by adding Taq buffer to 1× final concentration, 100 nmol dATP and 2.5 U Taq polymerase (Promega) to the reaction. The incubation was carried out at 74°C for 2 h before purification and subcloning.

The primers used to clone the fragments expressing Bru-3-derived proteins A–H were as follows: A, 5′-atggttcatattattgaattggtc-3′ and 5′-gcatttagatcgcaagctgttc-3′; B, 5′-atggttcatattattgaattggtc-3′ and 5′-caaactatgccgggcgcatcc-3′; C, 5′-gttggcatgctcagcaaacaa-3′ and 5′-caaactatgccgggcgcatcc-3′; D, 5′-gttggcatgctcagcaaacaa-3′ and 5′-aatctcttcatctaccatttg-3′; E, 5′-tccagcctggtcgtcaaatacgcc-3′ and 5′-aatctcttcatctaccatttg-3′; F, 5′-tccagcctggtcgtcaaatacgcc-3′ and 5′-ggatgccagtcgaccctattaa-3′; G, 5′-ccccaggagtttggcgacgcc-3′ and 5′-ggatgccagtcgaccctattaa-3′; H, 5′-atggttcatattattgaattggtc-3′ and 5′-ggatgccagtcgaccctattaa-3′.

To subclone the HA-tagged CUG-BP cDNA, basically the same protocol was used as above except that the annealing temperature was 55°C with the following primers 5′-ggatccggatccggatccatgaacggcaccctggaccac-3′ and 5′-ctcgagctcgagtctagattaagcgtaatctgggacgtcgt atgggtagtagggctttgctgtcattctt-3′.

For expression in S2 cells, the His-tagged Bru-3 fragment from plasmid pTrc-HisTOPO-Bru-3 was subcloned between the EcoRI and the blunt-ended KpnI sites of the eukaryotic plasmid pMT/V5-HIS (Invitrogen). The following procedure was used. pTrc-HisTOPO-Bru-3 was digested by NcoI at 37°C for 2 h. After phenol/chloroform extraction, the NcoI extremities of the Bru-3 fragment were filled up with 5 U T4 DNA polymerase at 37°C for 5 min in the following buffer: 0.1 mM each dNTP, 33 mM Tris–acetate pH 8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 0.1 mg/ml BSA. After a new phenol/chloroform extraction, the Bru-3 fragment was cut out from the linearized plasmid with EcoRI and purified in low melting point agarose. Similarly, the pMT/V5-HIS expression plasmid was first digested with KpnI, then the extremities were filled in with T4 DNA polymerase prior to EcoRI digestion. Finally, the His-tagged Bru-3 fragment was subcloned into pMT/V5-HIS for use in transfection experiments.

Protein expression and extraction from bacteria and S2 cells. Competent TOP 10 bacterial cells were transformed with the Bru-3 derived constructs as indicated by the manufacturer (Invitrogen). Cells were grown in LB medium containing ampicillin (50 μg/ml) and glucose (0.025 mM) at 37°C to an A_{600} of 0.6, induced with 10 mM isopropyl-β-D-thiogalactopyranoside and harvested 4 h later. After centrifugation the cells were resuspended in extraction buffer (50 mM Tris–HCl pH 7.8, 150 mM NaCl, 0.25% NP40) with protease inhibitors [leupeptin, antipain, pepstatin, aprotinin at 5 μg/ml, benzamidine and phenylmethylsulphonyl fluoride (PMSF) at 1 mM] and lysed by sonication.

Schneider (S2) cells were grown in SF-900 II medium with L-glutamine (Gibco) supplemented with penicillin and streptomycin (100 U/ml). Cells were passed every 3 days to maintain exponential growth. One day before transfection, 4×10^6 cells/well were seeded in 2 ml growth medium in six-well plates. Transfection of 0.8 μg pMT/V5-HIS Bru-3 was carried out with 25 μl of Effectene using the manufacturer's protocol (Qiagen). Six hours after transfection, $CuSO_4$ was added to the growth medium to obtain a 0.5 mM final concentration. The transfected S2 cells were harvested 2 days after transfection. After centrifugation, the cells were resuspended in the same extraction buffer as used for the TOP 10 bacterial cells and lysed by sonication. After sonication, glycerol was added to 20–40% final concentration.

Drosophila protein extracts. Hand dissected ovaries and embryos were used. The embryos were collected overnight and treated with bleach to remove the chorions. Protein extracts were prepared by homogenizing the ovaries and the embryos in ice-cold extraction buffer (50 mM Tris–HCl pH 7.8, 150 mM NaCl) with a cocktail of protease inhibitors (leupeptin, antipain, pepstatin, aprotinin at 5 μg/ml, benzamidine and PMSF at 1 mM). After a 10 min centrifugation at 12 000 *g* at 4°C, glycerol was added to obtain a 20% final concentration.

RNA–protein complex analysis

UV cross-linking assay and western blot analysis. The cross-linking procedure was adapted from a published protocol (30). The reaction was carried out with 40 μg *Drosophila* protein extract (ovarian, embryonic and from S2 cells) or 20 μg bacterial extract in a buffer containing 10 mM HEPES pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl with 1 mg/ml tRNA. Aliquots of 100–200 fmol ^{32}P-labelled RNA probe were added and the incubation carried out at room temperature for 15 min. After UV cross-linking at 1 J for ~7 min in a BLX type Bio-link apparatus (Bioblock Scientific), the samples were treated with 20 μg RNase A at 37°C for 30 min and submitted to electrophoresis on a 10–12% SDS–polyacrylamide gel. After transfer to nitrocellulose membrane (Protran; Schleicher & Schuell) and PhosphorImager exposure, we proceeded to western blot analysis. After saturation with 5% milk in Tris-buffered saline (TBS) (10 mM Tris–HCl pH 7.5, 140 mM NaCl), the membrane was incubated at 4°C overnight in TBS with a rabbit antiserum directed against the histidine epitope (1/1000[e]). After several washes in TBS, the membrane was incubated for 1 h at room temperature with alkaline phosphatase-coupled secondary anti-rabbit IgG antibody (1/10 000 in TBS). The secondary antibody and the NBT-BCIP revelation kit were from Promega.

Band shift assay. The electromobility shift assay was essentially as previously described with slight modifications (31). Aliquots of 40 μg protein extract were incubated with ~20 fmol denatured RNA probe (5×10^4 c.p.m.) for 15 min on ice, in 15 μl of reaction buffer (25 mM HEPES–KOH pH 7.9, 0.01% NP40, 1 μM $ZnSO_4$, 100 mM KCl, 1 μM $MgCl_2$, 1 μM $MnCl_2$, 1 μg tRNA and 20 U RNasin). Whenever required, unlabelled competitor RNA was added at a 500-fold excess. Heparin (10 μg) was then added on ice 5 min before loading. The mix reactions were separated on a pre-electrophoresed 5 or 7% polyacrylamide gel (19:1 acrylamide:bisacrylamide) in 0.5× TBE buffer. The gel was dried under vacuum and the samples visualized with a PhosphorImager (Storm 820; Molecular Dynamics).

For supershift assays, lysates were incubated with an anti–His_6 rabbit antiserum (1/15^e) for 5 min on ice prior to the addition of RNA probes.

Northwestern blotting. The ovary and embryo protein extracts were separated by 10% denaturating SDS–PAGE and electrotransferred to nitrocellulose membrane. Membrane-bound proteins were renatured overnight at 20°C in 10 mM HEPES pH 7.9, 40 mM KCl, 5% glycerol, 0.2% NP40, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT and 5 mg/ml BSA as previously described (32). After renaturation, the membrane was incubated at 30°C for 2 h with EDEN RNA probe (1.5×10^5 c.p.m./ml) in binding buffer (10 mM HEPES pH 7.9, 150 mM KCl, 8% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol and 50 μg/ml tRNA) followed by a 20 min RNase A digestion (25 μg/ml) at 37°C. The membrane was washed twice with 10 mM HEPES pH 7.9, 150 mM KCl, 8% glycerol and 5 mM $MgCl_2$ and submitted to autoradiography.

RESULTS

Sequence comparison and phylogenetic analysis of EDEN-BP related proteins

We have shown in our previous work that the EDEN-dependent translational repression of maternal mRNAs is conserved between *Drosophila* and *Xenopus* (29). This means that a *trans* acting factor exists in *Drosophila* to account for this repression. Bruno, a *Drosophila* translational repressor specific for the oocyte, was considered as the EDEN-BP orthologue. However, because translational repression of an EDEN-containing transgene is maintained in the embryo, we hypothesized the existence of another protein similar to EDEN-BP which could be the genuine EDEN-BP orthologue (29).

The availability of the *Drosophila* genomic sequence prompted us to carry out a blast search for a previously unidentified gene the sequence of which makes it a good candidate for being the EDEN-BP orthologue. In fact, two Bruno paralogues have been identified, Bru-3, a 422 amino acid encoding cDNA (LD31834), and Bru-2, a 737 amino acid encoding cDNA. The gene encoding Bru-2 maps at 33E1-E3 on the left arm of the second chromosome, near Bruno, while Bru-3 is encoded by a gene which maps at 70B5-C2, on the left arm of the third chromosome. In order to gain more insight into the EDEN-BP orthologues/paralogues in insects, we also searched the *Anopheles* genome sequence for similar sequences. Our scope was not to carry out an extensive analysis of the 117 RRM-containing proteins identified in *Drosophila* (33). We restricted our sequence comparison and phylogenetic analysis to the sequences we have considered to be relevant to our search for the EDEN-BP orthologue. Therefore, we compared EDEN-BP, CUG-BP, Bruno, Bru-2, Bru-3 and the two closest sequences identified in *Anopheles gambiae*, ebiP2417 and ebiP5501. The domain organization of the seven sequences considered here is shown in Figure 1A. Among these proteins only Bru-3 and the *Anopheles* ebiP2417 have two RRMs, due to a shorter N-terminus, while all the others have three RRMs. Sequence comparisons showed that Bru-3 and ebiP2417 have lost the first RRM. It is noteworthy that these two amino acid sequences share 87% similarity and 80% identity (Fig. 1A), while Bruno and ebiP5501 share 76% similarity (data not shown). Surprisingly, there is no Bru-2 equivalent in *A.gambiae*. All these proteins display alanine stretches of various lengths and a lsm was found specifically in EDEN-BP, CUG-BP, Bru-3 and its *Anopheles* orthologue (described later in this work).

In order to illustrate how these proteins relate to each other we carried out a phylogenetic analysis based on a Clustal-X multiple alignment of the above sequences. The resulting unrooted tree clearly suggested that the *Drosophila* Bruno and Bru-3 sequences were orthologous to the *Anopheles* sequences ebiP5501 and ebiP2417, respectively. Bru-2 was on a different branch. This tree was validated by strong bootstrap values. It is noteworthy that the genetic distance between Bru-3 and ebiP2417 is comparable to the distance between EDEN-BP and CUG-BP, while Bruno and its *Anopheles* orthologue ebiP5501 seem to be more divergent (Fig. 1B).

The *Drosophila* endogenous EDEN binding factor is a 45 kDa protein

To determine which of the endogenous *Drosophila* Bruno paralogues is capable of binding the EDEN element and therefore is the likely candidate for EDEN-dependent translational repression, we prepared protein extracts from 0–12 h embryos and ovaries. Three different methods were used in an attempt to identify the protein(s) involved in the binding of the EDEN element (Fig. 2).

In the first experiment, the protein extracts were incubated with a radiolabelled RNA probe and submitted to electrophoresis under non-denaturing conditions in order to reveal all the complexes formed. The results of an electrophoretic mobility shift assay (Fig. 2A) show clearly that two slow migrating bands were specifically formed in the presence of the ovarian and embryonic extracts. While the two complexes were efficiently competed out by an excess of cold EDEN RNA, they were poorly affected by a non-specific RNA probe used under the same conditions. The fast migrating complex (complex 2), which usually formed in both the ovarian and the embryonic extracts, seemed to be unstable; in this experiment the corresponding band was very weak with the embryonic extracts (Fig. 2A).

In order to identify the proteins involved in the complexes observed in the gel shift experiment, we submitted the RNA–protein complexes to UV cross-linking before analysis by SDS–PAGE and transfer to nitrocellulose membrane. To assess the specificity of binding, the extracts were also incubated with a non-specific RNA probe. Autoradiography of the membrane is displayed in Figure 2B. Interestingly, in endogenous extracts only a 45 kDa protein (p45) was involved in direct binding of the EDEN probe and this binding was specific as no signal at all could be detected when the extracts were incubated with the non-specific RNA probe. Moreover, the same protein is apparently involved in EDEN binding in both the embryo and the ovary. To confirm this result we carried out a northwestern experiment. In this experiment, the *Drosophila* extracts and the control *Xenopus* egg extracts were submitted to SDS–PAGE without prior incubation with the RNA probe and transferred to a nitrocellulose membrane as for a classical western blot. The membrane was then incubated with the radiolabelled RNA probe after a renaturation step and submitted to autoradiography (Fig. 2C). The northwestern

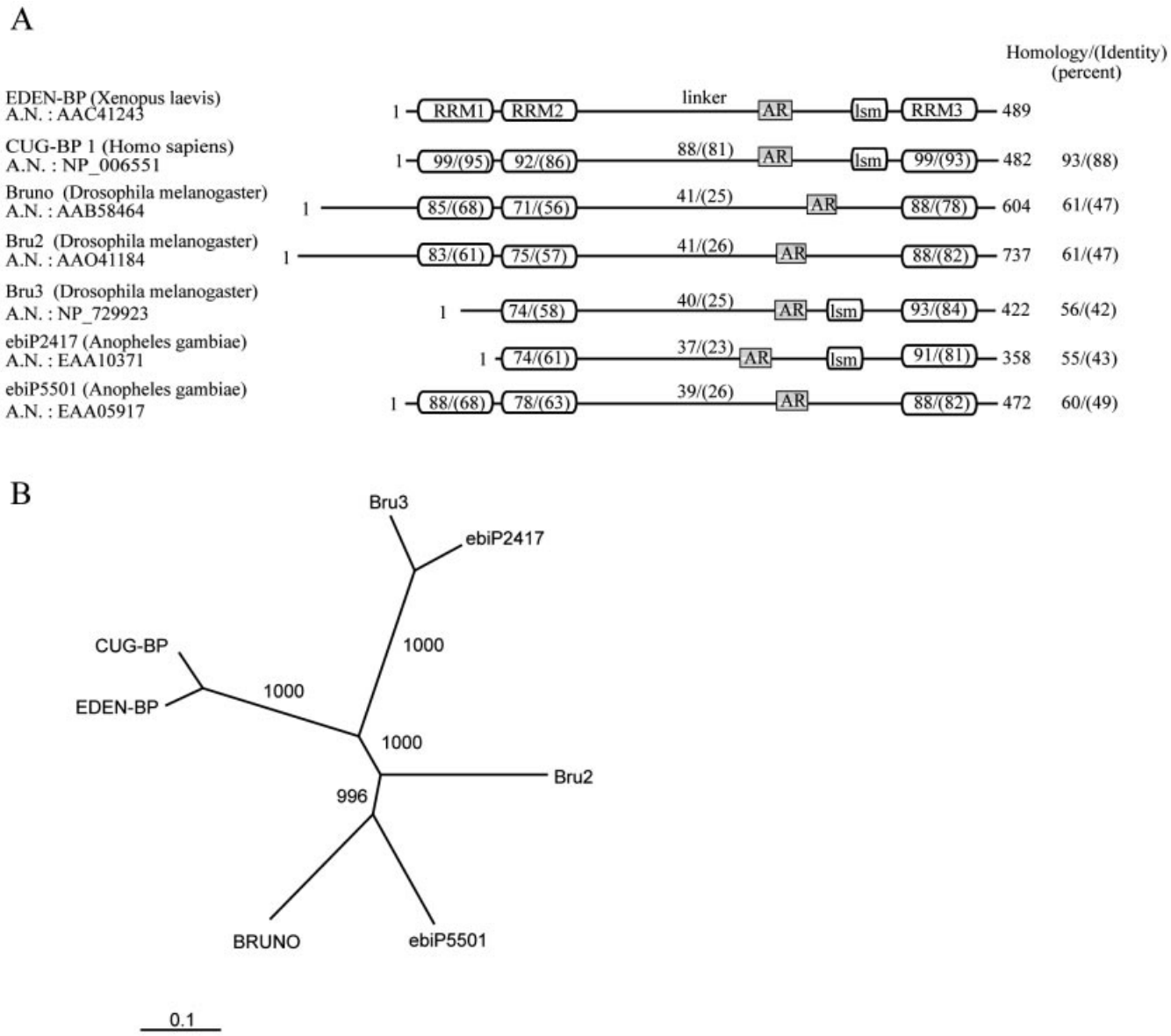


Figure 1. Sequence comparison and phylogenetic analysis. (**A**) Schematic representation of the conserved domains of the closest proteins to EDEN-BP in *Homo sapiens* (CUG-BP), *Drosophila melanogaster* (Bru-2, Bru-3, Bruno) and *Anopheles gambiae* (ebiP2417, ebiP5501). The numbers indicate the sequence similarity/identity (numbers in brackets) resulting from the tblastn search using EDEN-BP as the query sequence. Accession numbers (A.N.) are indicated for the different protein sequences. The boxes represent characteristic domains (RRM, RNA recognition motif; AR, alanine-rich region; lsm, linker-specific motif). The drawings are not to scale. (**B**) Unrooted phylogenetic tree based on the alignment of the above sequences. Bootstrap values and the divergence scale are indicated.

revealed a single EDEN binding activity at 45 kDa in both embryonic and ovarian protein extracts; this binding was specific as no signal was detected with the antisense probe (data not shown). As a control, *Xenopus* egg extracts were used under the same conditions. *Xenopus* EDEN-BP could be revealed at ~50 kDa. The high mobility bands are likely to be degradation forms of the protein (also seen in the *Drosophila* ovarian extracts). A band with a mobility slower than EDEN-BP was also observed with the *Xenopus* extracts. Given the specificity of our experimental conditions, it is unlikely that this band was due to some artefact in our experimental conditions. As a matter of fact, these bands were also found when analysing *Xenopus* proteins retained on an EDEN affinity column (9).

Taken together, all these experiments indicate that the binding of EDEN in endogenous protein extracts is highly specific. They point to a 45 kDa protein present in both ovaries and embryos. These experiments definitely rule out Bruno and Bru-2 as the EDEN binding activities, these proteins being expected to migrate at 67 and 75 kDa, repectively. Moreover, Bruno is restricted to the ovary (16). Therefore, the only protein likely to be the *Drosophila* EDEN binding factor is the

two RRM protein Bru-3, the sequence of which encodes a 422 amino acid protein which is compatible with migration at 45 kDa.

This result was unexpected, as Bru-3, as well as its *Anopheles* orthologue, has only two RRMs. Therefore, it was important to verify the EDEN binding capacity of Bru-3.

Bru-3 binds the EDEN element efficiently and specifically and acts as a homodimer

Bru-3 is encoded by a gene the size of which is unusually large, leading to the exons at first being misassigned as two different genes (CG 10046 and CG 12478). Expressed sequenced tags (ESTs) have been identified in ovaries, embryos, testis and adult heads, showing a highly complex tissue-specific splicing pattern, the embryonic and ovarian forms being the same. In the framework of our search for the EDEN-dependent translational repressor of maternal mRNAs, we focused on the maternal form. A full-length 2.6 kb cDNA (LD 31834) from the embryonic DGC collection was sequenced, allowing an unambiguous assignment of the 13 exons to one gene which spans at least 137 kb (Fig. 3A). We have verified the sequence reported for the LD 31834 clone and the size of the RNA by northern blot analysis (data not shown). Our experimental data are in good agreement with the data reported by the BDGP. The corresponding gene (accession no. CG 12478) is called Bru-3 on the basis of sequence similarities with Bruno. It maps at 70 B5-C2 in a genomic region where neither mutations nor P element insertions have been reported so far. In order to experimentally test the capacity of Bru-3 to bind the EDEN element, as hypothesized from an experimental analysis of the endogenous protein extracts, we expressed a His_6-tagged Bru-3 polypeptide in both bacteria and transiently transfected S2 cells. The binding activity was assessed either by UV cross-linking or gel shift experiments using either a $(UGUA)_{12}$ or a $(UG)_{15}$ *in vitro* transcribed ^{32}P-labelled RNA (Fig. 3B).

Cross-linking experiments allowed us to detect a radiolabelled band specifically with Bru-3-containing protein extract (as verified with an anti-His_6 antibody). This labelling was observed exclusively when the protein extract was incubated with the $(UGUA)_{12}$ probe, but not with the antisense $(UACA)_{12}$ probe nor with a non-specific RNA sequence expressed from the pBluescript linker. In an attempt to further characterize the binding specificity of Bru-3, we carried out the same experiment with a $(UG)_{15}$ probe. As a control, we used a protein extract from a CUG-BP-expressing bacterium. Interestingly, Bru-3 (45 kDa) binds the UG repeat with the same efficiency as CUG-BP (50 kDa). Binding of the UG

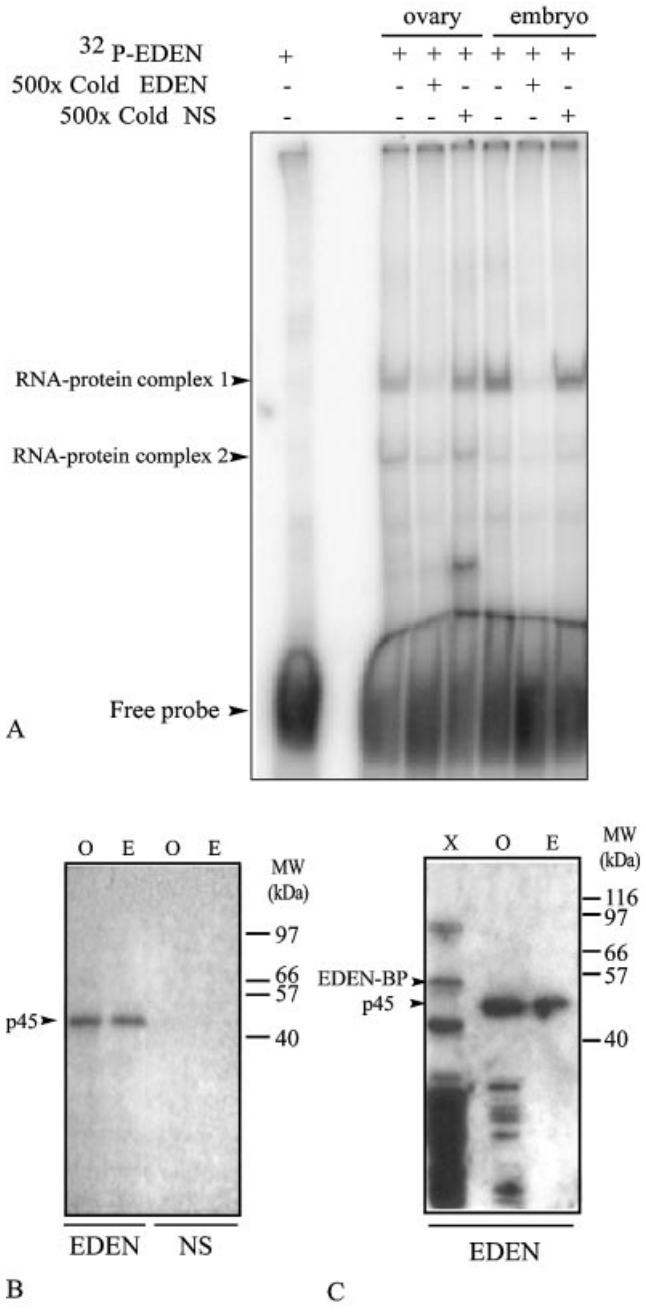
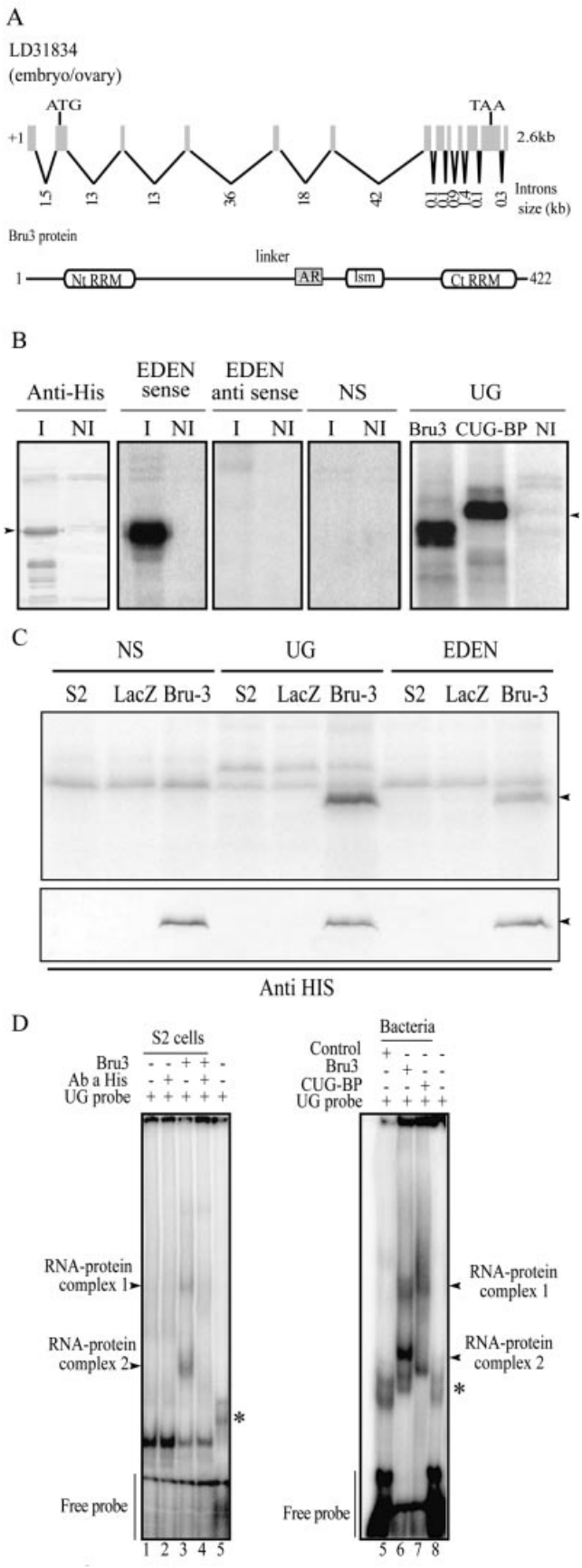


Figure 2. EDEN binding activity in *Drosophila* endogenous protein extracts. Embryonic and ovarian protein extracts were prepared as indicated in Materials and Methods. (**A**) Gel shift assays. Aliquots of 40 µg protein extract were incubated with 20 fmol ^{32}P-labelled EDEN RNA probe (~5 × 10^4 c.p.m.). This probe was used either undiluted or in a competition assay, diluted with a 500-fold excess of cold EDEN or non-specific RNA (NS) transcribed from the pBluescript polylinker. The first lane contains the EDEN RNA probe alone. The complexes formed were resolved by electrophoresis through a 7% native polyacrylamide gel (PAGE) and visualized with a PhosphorImager after gel drying. (**B**) UV cross-linking experiments. Aliquots of 40 µg *Drosophila* ovarian (O) and embryonic (E) extracts were incubated with 100 fmol (0.25–0.75 × 10^4 c.p.m.) EDEN RNA probe or with a non-specific RNA sequence and UV cross-linked. After RNase A treatment, they were submitted to SDS–PAGE separation and transferred onto nitrocellulose membrane. The UV-induced label transfer was visualized with a PhosphorImager. Molecular weights are indicated (MW). A p45 protein was detected specifically with the EDEN and not with the non-specific (NS) probe. (**C**) Northwestern blot analysis of *Drosophila* and *Xenopus* protein extracts. Aliquots of 40 µg *Xenopus* egg (X) and *Drosophila* ovarian (O) and embryonic (E) protein extracts were separated by 10% SDS–PAGE and electrotransferred to nitrocellulose membrane. After protein renaturation, the membrane was incubated with the EDEN RNA probe (1.5 × 10^5 c.p.m./ml). The EDEN binding activities were revealed by exposing the membrane to X-ray film. In *Drosophila*, a p45 protein binds the EDEN probe. The *Xenopus* EDEN-BP was revealed at ~55 kDa as well as other proteins in good agreement with the previously published data (9). Some degradation occurred in both the *Drosophila* and *Xenopus* extracts.



repeat and EDEN are highly specific as antisense EDEN or an RNA sequence expressed from the linker of the Bluescript vector failed to bind these proteins under the same experimental conditions (Fig. 3B). The results obtained with the same Bru-3 recombinant protein expressed in S2 cells are in perfect agreement with the results described above. The Bru-3 recombinant protein specifically recognizes EDEN and a UG repeat probe (Fig. 3C). When cross-linking was carried out with a non-specific probe, Bru-3 expressed in S2 cells failed to bind. Presence of the protein was assessed by western blot (as shown in the lower part of Fig. 3C). All these data indicate that Bru-3 is able to bind EDEN and UG repeat probes whether it is expressed in bacteria or *Drosophila* cells.

The binding pattern of Bru-3 was tested under native conditions using gel shift experiments. We used two types of protein extracts. Extracts from *Drosophila* S2 cells transfected with His-tagged Bru-3 and bacterial extracts expressing the same Bru-3 recombinant protein. Given the high binding affinity of the $(UG)_{15}$ repeat, it was used as the RNA probe in these experiments. We could detect a mobility shift of the $(UG)_{15}$ probe only when incubated with Bru-3-containing extracts and not with regular S2 extracts. A non-specific RNA probe expressed from a portion of the pBluescript linker was not shifted under the same conditions (data not shown). Two complexes were detected, the formation of which was competed out by a His_6 tag antibody (Fig. 3D, left). Therefore, both complexes contain the Bru-3 protein. It is likely that complex 2 contains a monomeric form while the larger complex 1 is an oligomeric form. As the protein extracts

Figure 3. Analysis of Bru-3 RNA binding specificity. (**A**) Exon disposition of the cDNA clone LD 31834/Bru-3 and schematic representation of its protein sequence. The boxes represent characteristic domains (RRM, RNA recognition motif; AR, alanine-rich region; lsm, linker-specific motif). (**B**) Western blot analysis and UV cross-links with bacterial recombinant Bru-3. Protein extracts (20 μg) from induced (I) or non-induced (NI) Bru-3 or CUG-BP transformed bacteria were incubated with 100 fmol different RNA probes and treated as in Figure 2. The following probes were used: sense EDEN $(UGUA)_{12}$; antisense EDEN $(UACA)_{12}$ and $(UG)_{15}$. The non-specific probe (NS) is a 60 nt long RNA sequence expressed from the Bluescript linker. The western blot was revealed with an antiserum which recognizes the $(His)_6$ tag. (**C**) Western blot analysis and UV cross-links with Bru-3-containing S2 cell protein extracts. The experimental conditions are as indicated above with the exception of the amount of protein extract (40 μg). The arrowhead indicates recombinant Bru-3 as revealed by a specific RNA probe (upper part of the panel) and the antibody (lower part). The NS, EDEN and UG probes are as indicated above. The controls are protein extracts from non-transfected S2 cells (S2) and *LacZ* transfected cells (*LacZ*). The slower migrating bands are not relevant to Bru-3 as they are present in extracts of both non-transfected cells and *LacZ* transfected cells. (**D**) RNA band shift and supershift assays. Aliquots of 60 μg Schneider S2 cell protein extracts (left) or 20 μg bacterial protein extracts (right) were used in these experiments. These extracts contain or not His_6-tagged Bru-3 or CUG-BP as indicated in the figure. They were incubated with 20 fmol $(UG)_{15}$ RNA probe. The rabbit anti-$(His)_6$ antiserum was used at a $1/15^e$ dilution. The secondary anti-rabbit antibody coupled to alkaline phosphatase and the revelation kit were from Promega. The control was a $(His)_6$-tagged β-galactosidase-containing bacterial extract. The complexes formed were resolved on a 5% native PAGE and visualized with a PhosphorImager after gel drying. The position of free probe is indicated as well as the positions of the specific complexes formed. The asterisk indicates a non-specific shift observed upon incubation of the probe in the gel shift assay buffer (last lanes of left and right panels). It is the only shift observed when the probe was incubated with the control bacterial extract (right panel, first lane).

used in this experiment were from *Drosophila* S2 cells, it was difficult to conclude whether it is a homo-oligomer or a hetero-oligomer formed with a protein partner present in S2 cells. To rule out this possibility, we carried out a similar experiment using the Bru-3-containing bacterial protein extracts used in the cross-linking experiments. As observed in Figure 3D (right), two RNA–protein complexes were formed with the $(UG)_{15}$ probe, displaying a similar migration pattern to those observed with protein extracts from S2 cells. As a control we used human CUG-BP, with which, similarly, two complexes were formed. The formation in bacteria and S2 cells of the same slowly migrating complex the size of which is comparable to the CUG-BP-containing complex is consistent with formation of a homodimer. In fact, the presence in both bacteria and S2 cells of a Bru-3 protein partner of the same size is unlikely. The slight RNA mobility shift observed even in the absence of protein extracts or in the presence of control bacterial protein extracts (expressing β-galactosidase) was due to either non-specific complex formation with some component of the incubation buffer or some RNA secondary structure.

In conclusion, the cross-linking experiments and the gel shift assays demonstrate that Bru-3 could bind specifically and efficiently to the same RNA targets as *Xenopus* EDEN-BP and human CUG-BP, despite the presence of only two RRMs. Moreover, a homodimer was formed by Bru-3 and CUG-BP (our data), which is in agreement with the data reported for *Xenopus* EDEN-BP (34).

The Bru-3 linker region and C-terminal RRM are the minimal requirements for RNA binding

We assumed that it was of interest to map the minimal Bru-3 sequence requirement in order to shed light on the molecular basis of efficient EDEN binding by Bru-3 despite the presence of only two RRMs in the protein. Seven domains of the Bru-3 cDNA were cloned in the same pTrcHis-TOPO vector as used for the complete Bru-3 ORF. All the polypeptides produced bore a His_6 tag at their N-terminal end, within a 4 kDa vector-expressed sequence (drawn in Fig. 4A). This allowed us to verify production of the truncated protein with an anti-Tag antibody by western blot. This western blot is quantitative, as all the polypeptides are similarly recognized by the antibody as they all have the same epitope at the same position in the chimeric protein.

The seven truncated domains and the Bru-3 protein were similarly produced in bacteria. The bacterial extracts were incubated in the presence of a ^{32}P-labelled $(UG)_{15}$ probe, UV cross-linked and submitted to electrophoresis on polyacrylamide gels under denaturing conditions and transferred to nitrocellulose. After autoradiographic exposure of the membrane, the His_6-containing polypeptides were revealed with the anti-Tag antibody. The results are shown in Figure 4B. In the left part of Figure 4B, the cross-linking experiment reveals that efficient binding was observed only with the non-truncated protein (lane H). However, some binding to the RNA probe occurred when the polypeptide containing the linker region and the C-terminal RRM was used in the experiment (lane F). However, this binding is far less efficient than that observed with the entire protein (lane H, left). This comparison was possible as similar amounts of the truncated proteins were present in the sample, as revealed by the same antibody (lanes F and H, right). Given the difference in the binding efficiency obtained with the minimal domain and the entire protein, one can assume that the different domains of this modular protein act synergistically for optimal RNA binding. It is noteworthy that while some binding was achieved with a combination of the linker and the C-terminal RRM, a combination of the N-terminal RRM and the linker failed to bind the RNA probe. We think that the C-terminal part of the linker is intimately involved in some higher order organization with the C-terminal RRM. This assumption deserves further attention.

Evidence for a conserved motif within the divergent linker region

Little attention is usually paid to the so-called linker region in the analysis of RRM proteins as these regions are usually divergent and their interest is less obvious than the highly conserved RRM domains. Three facts prompted us to look more carefully at the linker sequence. First, the capacity of Bru-3 to efficiently and specifically bind a synthetic EDEN and the $(UG)_{15}$ RNA probes despite the presence of only two RRMs is intriguing. Second, the minimal sequence required for this binding includes the linker and the C-terminal RRM; as shown above, a combination of the N-terminal RRM and the linker did not display any RNA binding. Third, despite a similar overall sequence identitity between EDEN-BP and the three related *Drosophila* sequences, only p45 was shown to bind EDEN; this suggests the presence of some structural component which could account for the specificity of Bru-3, EDEN-BP and CUG-BP for the same RNA sequence.

To gain some insight into the molecular basis of the properties of Bru-3, we carried out a careful manual sequence alignment between the linker regions of the proteins analysed in the present work. Surprisingly, a very well conserved motif could be identified within the divergent region. This hydrophobic and aromatic amino acid rich domain was found specifically in the linker regions of CUG-BP, EDEN-BP and the insect orthologues *Drosophila* Bru-3 and *Anopheles* ebiP2417 (Fig. 5A). Outside this domain, the insect and vertebrate sequences are highly divergent. It is noteworthy that neither Bru-2 nor Bruno nor its *Anopheles* paralogue ebiP5501 have this 35 amino acid lsm. We hypothesized that the lsm is a signature of a set of RRM proteins functionnally related to EDEN-BP. Indeed, the other vertebrate proteins in which this motif was found share 90% identity with EDEN-BP/CUG-BP and can therefore be considered as genuine orthologues (Fig. 5B). Interestingly, this motif was found to be conserved to a lesser extent in the etr-3 subgroup of proteins (data not shown).

To gain some insight into the function of this domain, we carried out a bioinformatic analysis using different threading methods. These methods are especially useful for analysing sequences with low identity scores. The relevance of the sequence–structure alignments was then evaluated at the 3-dimensional (3D) level. The Bru-3 domain, which spans amino acids 262–328, was used as the query sequence. Various 3D models were built using the TITO, SCWRL and MODELLER 6.2 programs (24–26) and then validated using the Verify3D and PROSA programs (27,28). Only α+β structures seemed compatible at the 3D level. The most significant score was obtained for a sequence–structure

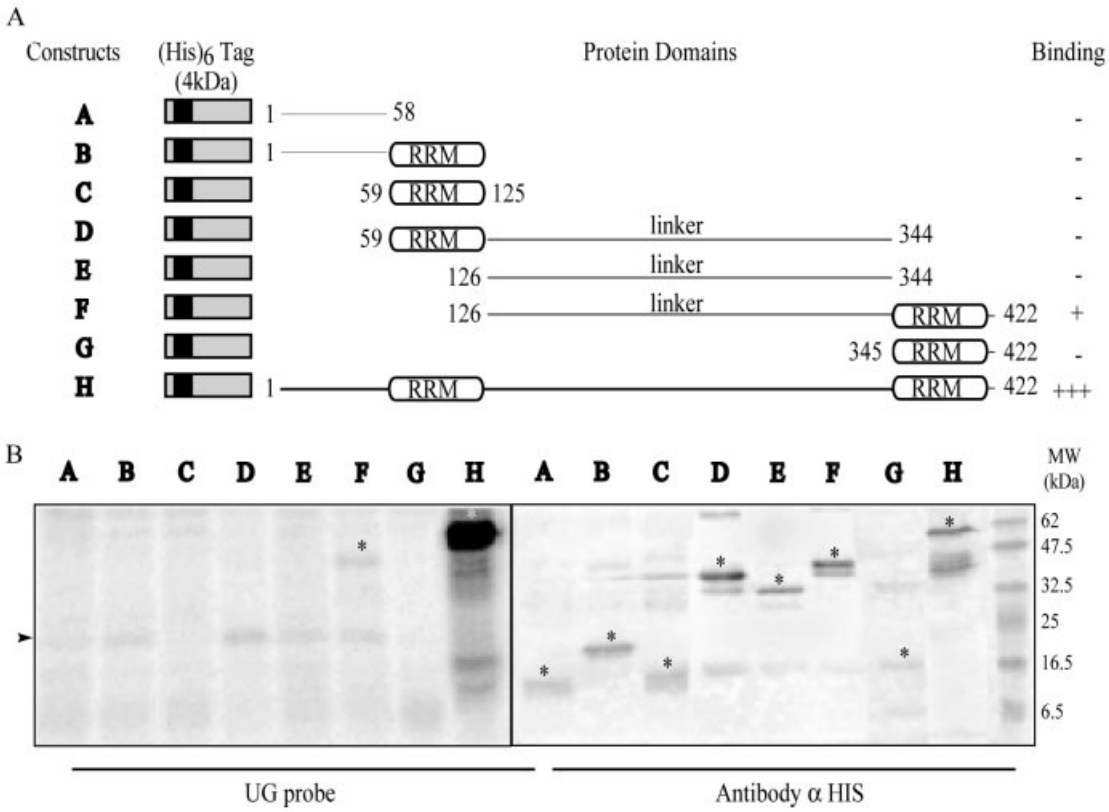


Figure 4. Analysis of RNA binding affinity of various truncated Bru-3 constructs. (**A**) Schematic representation of the truncated recombinant proteins. All the constructs bear a 4 kDa non-specific N-terminal peptide (grey box) with a His_6 tag (black box). The position of the RRMs is indicated by a white box; the amino acid boundary is relative to the wild-type protein. The binding affinity is given by the number of (+) signs. (**B**) UV cross-linking assay coupled with western blot analysis. Bacterial extracts containing the various constructs were incubated with a $(UG)_{15}$ RNA probe as indicated in the legend to Figure 3. After SDS–PAGE and transfer to nitrocellulose membrane, the transferred label was visualized using a PhosphorImager (left). The membrane was then treated with an antiserum which recognizes the epitope tag, under the conditions described in the legend to Figure 3 (right). The Bru-3-derived polypeptides are indicated with an asterisk; as shown, similar amounts of recombinant Bru-3-derived polypeptides were loaded. The arrowhead points to a non-relevant bacterial RNA binding protein.

alignment with a bacterial protein (PDB1EAY) that adopts a βαββαβ fold (Fig. 5C). The statistical significance of the structural alignment (the FUGUE Z-score is 3.86, despite a sequence identity of only 14%) suggested a certainty of >90%. The pseudo-energy computed by TITO and then by PROSA on the model produced by SCWRL suggested that a similar fold might be adopted by the lsm-containing region. As the recognized fold was observed in RRM domains, the potential presence of a cryptic RRM was further evaluated using as a template the *Drosophila* sex-lethal RRM–RNA co-crystal, the 3D structure of which is known. The proposed alignment was slightly refined by visual inspection of the corresponding structure and other related RRM structures (5,18,35). Analysis of the deduced common core highlighted the presence of conserved and buried hydrophobic residues likely stabilizing the overall fold. The possible impact of the numerous proline residues present in the lsm sequence (~20%) was carefully checked by a survey of their position in the structure. Most of these residues occurred in loops or at the beginning of secondary structure (especially true for the second predicted α-helix, which is flanked by a stretch of three prolines at its N-terminus). Using the structure of the sex-lethal RRM (accession no. PDB1B7F) as a template, a satisfactory 3D model was obtained as assessed by PROSA (-0.67) and Verify3D (0.42). These values were rather good considering the low sequence identity (~14%) and the particular amino acid composition of the lsm region.

Supplementary information including a detailed report of the results can be found at http://www.infobiosud.cnrs.fr/bioserver/LSM/suppl.html.

DISCUSSION

The endogenous *Drosophila* EDEN binding factor is a 45 kDa protein (p45)

We showed in our previous work that the mechanism of EDEN-dependent translational repression is conserved between invertebrates such as *Drosophila* and vertebrates such as *Xenopus* (29). The likely *Drosophila* candidate mediating this translational repression was Bruno, a factor first shown to repress the translation of *oskar* mRNA by means of binding to a specific sequence named a BRE (Bruno-responsive element) (15). Not only does the BRE show some similarity with EDEN but Bruno itself shares 50% similarity with EDEN-BP (9,16). Despite these similarities, our data and data published earlier

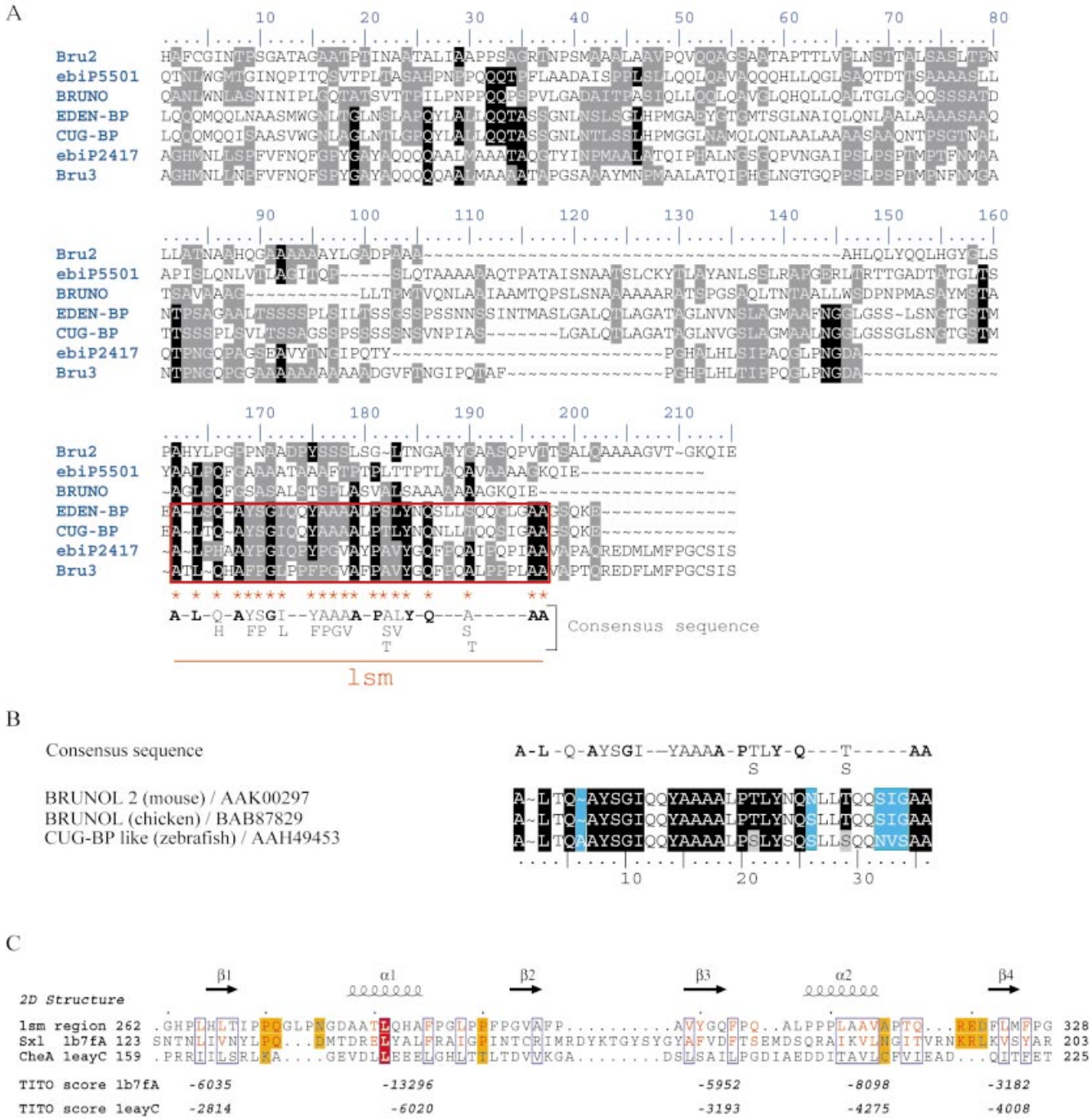


Figure 5. Sequence and secondary structure analysis of the Bru-3 linker region. (**A**) Sequence comparison of the linker region of the Bruno-like proteins analysed in this work. The MEME program allowed the identification of a linker-specific motif (lsm), the alignment was optimized with Bioedit. Grey and black shadings highlight similar and identical residues, respectively, using a 50% consensus (for all the proteins analysed). The motif was most conserved between EDEN-BP/CUG-BP and their insect orthologues (*Drosophila* Bru-3 and *Anopheles* ebiP2417). The perfectly conserved positions in the consensus sequence derived from the alignment of these four proteins are in bold. (**B**) Result of a MEME search in the databases for a lsm motif. Grey and black shadings highlight similar and identical residues, respectively, using a 50% consensus. The blue shading indicates the less conserved positions. (**C**) Compilation of the TITO-MODELLER outcome. A sequence spanning amino acids 262–328 was submitted to a sequence–structure comparison as described in Materials and Methods. TITO scores are shown on the figure (below the alignment). The lower the score is, the more likely that there is structural homology. The best structural alignment was obtained with a RRM. Sequence similarities according to the RISLER matrix are shown in boxes (letters in red for similarities restricted to lsm and sxl RRM) using ESPRIPT (38). Observed secondary structures in the 3D model of the lsm region are drawn above the alignment (38). 1b7fA corresponds to the first RRM of the sex-lethal protein (accession no. P19339) and 1eayC corresponds to the cheA bacterial protein (accession no. NP_416402). The detailed structural analysis is available at http://www.infobiosud.cnrs.fr/bioserver/LSM/suppl.html.

argue against EDEN and BRE acting through the same pathway (29). We confirm in this work that, indeed, Bruno does not bind EDEN in endogenous *Drosophila* protein extracts. The EDEN binding factor is a 45 kDa protein, as

shown not only by the cross-linking experiments but also by the northwestern analysis. Moreover, these experiments and the electrophoretic mobility shift assay, which allows analysis of the complexes under non-denaturing conditions, favour a protein which is both ovarian and embryonic, whereas Bruno is restricted to the *Drosophila* oocyte.

Bru-3, one of the two Bruno paralogues, is a specific EDEN binding factor

Two paralogues of Bruno, Bru-2 and Bru-3, were identified as a result of the availability of the *Drosophila* genome and cDNA sequences. A phylogenetic analysis showed that while Bruno and Bru-3 have *Anopheles* orthologues (ebi P5501 and ebi P2417, respectively), Bru-2 seems to be specific to *Drosophila*. It is also noteworthy that insects have evolved a two RRM EDEN-BP-related protein, as not only Bru-3 but also its *Anopheles* orthologue have a single N-terminal RRM. Surprisingly, the only Bruno paralogue likely to be the EDEN binding factor is the 422 amino acid polypeptide Bru-3. Bru-2 encodes a 737 amino acid polypeptide, therefore its mobility is expected to be ~75 kDa (slower than the 67 kDa Bruno). All our experiments are consistent with the presence of a single endogenous EDEN binding protein the size of which is ~45 kDa, which rules out not only Bruno but also Bru-2 as candidates for mediating translational repression by means of EDEN binding. The capacity of Bru-3 to bind EDEN was verified and, indeed, this binding is specific. Moreover, it binds $(UG)_{15}$ repeats preferentially. No binding to $(UA)_{15}$ repeats was observed under the same experimental conditions (not shown), as reported previously for EDEN-BP/CUG-BP-related proteins (36). It is interesting that Bru-3 binds EDEN as a dimer, as was shown for EDEN-BP (34).

To account for the above data, our hypothesis is that RRM1 of the three-RRM-containing proteins is dispensable provided that the linker region can fulfil its RNA binding activity.

The Bru-3 linker region is essential for specific RNA binding

Specific and efficient RNA binding requires the whole Bru-3 protein, presumably because the different domains of the protein act synergistically. However, mapping of the RNA binding domain showed that a polypeptide containing the linker region and the C-terminal RRM is the minimal requirement for binding activity. Many authors have reported necessity of the linker region in RNA binding, whereas two RRMs are usually sufficient for the RNA binding activity of three-RRM proteins. As an example, Bruno was cloned through BRE binding to a polypeptide which spans part of the linker region and the C-terminal RRM (16). *Xenopus* EDEN-BP requires the N-terminal RRMs and at least part of the linker region (34). However, no published work so far has reported how efficient this binding is. Surprisingly, despite the admitted importance of the linker region in the binding activity of RRM proteins, more attention was given to the highly conserved RRM domains.

Our hypothesis was that the molecular basis of the binding specificity of Bru-3 and EDEN-BP-related proteins should be sought in the linker region. The most striking feature in support of this hypothesis is the presence of a lsm specifically in EDEN-BP/CUG-BP, Bru-3 and its 87% similar *Anopheles* orthologue. It is noteworthy that this motif was not found either in Bruno or its *Anopheles* orthologue ebiP5501 nor in *Drosophila* Bru-2. It was found in a small number of vertebrate EDEN-BP/CUG-BP-related proteins, which showed ~90% identity. Interestingly, this motif is slightly divergent in etr-3 and other related proteins, despite their overall high sequence similarity to CUG-BP/EDEN-BP (7). In conclusion, the vertebrate proteins in which we could identify a lsm have a sequence identity such that they are certainly orthologues. Therefore, we consider the lsm as a signature of EDEN-BP/CUG-BP orthologues.

Strikingly, the lsm is always located in the same region of the linker, between the alanine-rich domain (11 Ala residues in Bru-3) and the C-terminal RRM. This led us to assume an important structural role for this motif. Indeed, sequence–structure comparisons predicted that the 262–328 amino acid domain which contains lsm might adopt a structure similar to that of a RRM domain. As this conservation is predicted from a weak sequence–structure comparison, it should be interpreted cautiously. However, this gives future directions to test experimentally which of the linker residues are important for RNA binding and whether the lsm is indeed part of a cryptic RRM.

One attractive hypothesis is that the linker region contains the most ancient RNA binding domain. However, this would have diverged to acquire target specificity while the topology necessary for binding RNA and/or interacting with specific partners would have been conserved. As the lsm was always found close to the C-terminal RRM, we hypothesize the existence of a larger topological domain which would include the lsm. Given our experimental data, it might be possible that the Bru-3 lsm and the C-terminal RRM organize in tandem and define the minimal requirement for specific RNA binding.

Can these structural features account for the failure of endogenous Bruno and Bru-2 to bind EDEN?

Despite the sequence similarity of Bruno and its two paralogues Bru-2 and Bru-3, the RNA binding specificity of these proteins in endogenous extracts is noteworthy. Moreover, the EDEN and BRE sequences share important similarity (9,15). Indeed, the action of BRE seems to be mediated exclusively by Bruno. To support this view, there is no functional redundancy for Bruno and its paralogues as the *arrest* mutations in the Bruno gene result in dramatic effects on oogenesis despite the presence of wild-type copies of Bru-2 and Bru-3 (16).

One explanation resides in the divergent regions, not only the linkers but also the N-terminal auxillary domains. These domains may play an important role in the binding specificity. In conclusion, it is likely that the specificity is achieved as a result of a combinatorial action of all the domains of these modular proteins (37).

This high specificity is confirmed by our data, as the EDEN element is specifically bound by the p45 protein, and deserves attention.

The biological role of the Bruno paralogues

It is likely that the three proteins act in the control of RNA translation. Some of the targets controlled by Bruno are known to be important for *Drosophila* oocyte formation. It is clear that in *Drosophila* the EDEN element induces translational repression of chimeric RNAs (29). The present work shows

that this is probably achieved through its binding to a p45 protein. The next step will be to formally demonstrate the action of Bru-3 as a translational repressor. The function of these proteins may not be restricted to translational repression. The first identified function for the human EDEN-BP orthologue, CUG-BP, is as a regulator of splicing (11). It is now clear that CUG-BP is also involved in the translational control of specific RNAs. In fact, CUG-BP is a multifunctional protein which shuttles between the nucleus and the cytoplasm depending on its phosphorylation status (11). Bruno is strictly cytoplasmic and specific for the oocyte, while Bru-2 ESTs have been identified only in embryonic RNA. As expected for an EDEN-BP/CUG-BP orthologue, Bru-3 is ubiquitous, as its RNA is expressed throughout development (our unpublished data). In addition, different ESTs were identified in adult heads and testis as the result of complex splicing of an unusually large gene. More work is needed to produce a complete description of the splicing pattern of this gene and antibodies against Bru-3 are necessary for a comprehensive description of the protein distribution throughout fly development. So far, two unsuccessful attempts have been made to raise antibodies against Bru-3. The pitfalls are the poor antigenicity of the protein and its high hydrophobicity, making its solubilization difficult. Also, no mutant alleles are available. A biological analysis requires antibodies and mutant alleles; developing these tools is our objective for the near future.

ACKNOWLEDGEMENTS

The LD31834 Bru-3 cDNA was from the Berkeley *Drosophila* Genome Programme. We thank J.Demaille, C.Ferraz and A.Bucheton for providing the clone and sharing information. Régis Meyer and Julia Roche are acknowledged for reading the manuscript. We thank J.M.Lemaître for providing the *Xenopus* extracts and B.Osborne and L.Paillard for discussions in the early phase of this work. This work has been partly supported by research grants from the Association pour la Recherche contre le Cancer (ARC 9932) and the Association Française contre les Myopathies(AFM) to O.A. J.D. was the recipient of a 3 year fellowship from la Ligue Nationale contre le Cancer and a 1 year ARC fellowship. G.L.M. is supported by the national PhD training programme (MENRT). N.E. was the recipient of an AFM fellowship.

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