Folding and binding activity of the 3′UTRs of *Paracentrotus lividus* bep messengers

Giovanna Montana[a], Elisabetta Sbisà[b], Daniele P. Romancino[a], Angela Bonura[a], Marta Di Carlo[a,*]

[a] *Istituto di Biologia dello Sviluppo, C.N.R., via Ugo La Malfa, 153, 90146 Palermo, Italy*
[b] *Centro di Studio sui Mitocondri e sul Metabolismo Energetico, C.N.R., via Amendola, 165/A, 0126 Bari, Italy*

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Abstract ***Bep*** **mRNAs are localized at the animal pole of** ***P. lividus*** **eggs. In the present communication the secondary structures of the 3′UTRs of the** ***bep1***, ***bep3*** **and** ***bep4*** **mRNAs are reported. The minimal lenghts of these regions required to bind the 54-kDa protein, previously shown to be involved in localization and anchoring of these RNAs, is estimated. Microinjection of the** ***bep3*** **3′UTR into egg shows that this RNA fragment is also able to become localized to one of the egg poles, as happens for the entire** ***bep3*** **RNA.**

Key words: A/V axis; *cis*-acting factor; RNA localization; 3′ Untranslated region

1. Introduction

Animal/vegetal axis determination in egg may require the asymmetric distribution of RNAs and proteins in the cytoplasm. While mRNA localization has been well documented in many systems the mechanism that generates restricted RNA distribution is less well understood. According to the observation of several investigators it seems that the localization process consists of different steps: formation of RNP particle, translocation of the particle to destination, anchoring to the cytoskeleton [1]. Translation of the localized mRNA finally brings about localization of the corresponding protein. The localization of RNA requires the presence of a *trans*-acting machinery and of a *cis*-acting sequence usually residing in the 3′UTR of the transcript [2,3]. These sequences should contain one or more localization signals. The recognition of a localization signal usually requires the capacity of RNA molecules that are single strand to fold into complex secondary structures. Furthermore such structures should contain one or more multiple binding-protein sites. Sequences located in the 3′UTR of some messengers and relevant for their localization have been identified both in *Drosophila* and in *Xenopus* [4–6].

All these identified 3′UTR sequences have been suggested to have an RNA binding motif for some protein. In the last few years searching for these proteic factors has permitted the identification of some of them both in *Drosophila* and *Xenopus* [7,8].

In sea urchins the localization process has not yet been studied because only recently three maternal mRNAs, called *bep1*, *bep3* and *bep4*, asymmetrically distributed in *P. lividus* egg have been identified [9,10]. These messengers encode for cell surface proteins that are involved in cell–cell interactions and may be relevant for the determination of the animal side of the sea urchin embryo [11,12]. Moreover it has been demonstrated that cytoskeleton is involved in their localization and that the 3′UTRs of these mRNAs are able to bind to a 54-kDa protein that we suggested to name LP54 (localizing protein) [14].

In this report we analyze the possibility that the 3′UTRs of *bep* mRNAs fold in secondary structures and identify an element in the 3′UTR sequence necessary for the binding to the 54-kDa protein. In addition, microinjection of an excess of 3′UTR transcripts into sea urchin eggs indicates a role of this *bep3* region in localization processes.

2. Materials and methods

2.1. Computer analysis

Preliminary multiple alignments were obtained by using PILEUP program [15] and then carefully revised manually in order to highlight nucleotide similarities present in the structures at homologous positions. PRETTYPLOT program was used to display boxed sequence alignments [14]. The output produced by PRETTYPLOT allows to visually single out the regions common to all the compared sequences and those with a similarity common only to some of the compared sequences. Secondary structures are predicted using the computer program FOLDRNA [15]. SQUIGGLES program was used for secondary structure representation [15].

2.2. Preparation of DNA constructs

The *Acc*I-*Xho*I subclone of *bep3* [10] was linearized by digestion at the *Acc*I site and incubated at different times with Bal31 enzyme. The deleted fragments obtained were subcloned into the appropiate sites of the Bluescript plasmid.

2.3. In vitro synthesis of transcripts

The *Acc*I-*Xho*I subclone of *bep3* [10] and the constructs descibed above were linearized by digestion at the *Xho*I site of the linker and transcription by T3 polymerase was carried out utilizing either the Riboprobe kit (PROMEGA) and [^{32}P]UTP for Northwestern assay, or the DIG-RNA Labeling kit (Boehringer) and DIG-UTP for microinjection, according to the manufacturer's instructions.

2.4. RNA binding assay

Northwestern analysis was carried out according to Montana et al. (1997) [14].

2.5. Microinjection in sea urchin eggs

Dejellied *P. lividus* unfertilized eggs [12] were microinjected essentially as described by McMahon et al. (1985) [17] with about 1500 molecules of *Acc*I-*Xho*I sense RNA, transcribed utilizing DIG-UTP. After fertilization the eggs were fixed in 4% paraformaldehyde and the

*Corresponding author. Fax: (39) (91) 6809548.
E-mail: di-carlo@ibs.pa.cnr.it

Abbreviations: *P. lividus*, *Paracentrotus lividus*

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exogenous RNA was detected by immunoassay using an anti-digoxigenin alkaline phosphatase conjugate (anti-DIG-AP) according to the manufacturer's instructions of the DIG Nucleic Acid Detection kit (Boehringer).

3. Results and discussion

3.1. 3′UTR bep RNA secondary structure predictions

We considered the possibility that the 3′UTRs of *bep1*, *bep3* and *bep4* are able to form similar secondary structures.

The 3′UTRs of *bep1* and *bep3* messengers are similar both in length and base composition (Fig. 1A), whereas *bep4* appears to be divergent. *Bep1* is 221 and *bep3* is 212 nucleotides long, whereas *bep4* is only 141 nucleotides long [10,16]. The first nucleotide of each sequence is the first one downstream from the stop codon.

With respect to base composition the U content is higher than the A content in *bep1* (U = 38%, A = 28%) and *bep3* (U = 36%, A = 33%), whereas in *bep4* the A content (45%) is much higher than the U content (27%). The G and C content is similar (C = 17%, G = 17% in *bep1*; C = 18%, G = 13% in *bep3*; C = 16%, G = 12% in *bep4*).

The program FOLDRNA [15] applied independently to each sequence predicts the optimal secondary structure, on the basis of minimization of free energy (−38.2 in *bep1*, −37.8 of *bep3*, and −12 in *bep4*). The three potential structures are shown in Fig. 1B. The 3′UTR of *bep1* recalls the specular image of the 3′UTR of *bep3*, and the 3′UTR of *bep4* might be considered as a portion of the others. The structures of the 3′UTRs of *bep1* and *bep3* RNA have five stems and three loops, whereas only two stems and two loops are present in the structure of the 3′UTR of *bep4*.

In *bep1* and *bep3* the two structures show small conserved sequence blocks in analogous positions with respect to the distribution of stems and loops (Fig. 1B; a,b).

The presence of similar secondary structure models suggests that a common RNA localization signal, able to recognize a specific protein, might be conserved in all the *bep* RNAs analyzed. Thus, this shared motif appears to act as a 'bep signal' recognized by an RNA-binding protein which mediates the association of these messages to cytoplasmic structures such as cytoskeleton and perhaps directs them, along a microfilament network, to their correct position within the cell.

3.2. Specific binding of 3′UTR sequences to a cytoplasmic protein

We have previously demonstrated that the fragment *Acc*I-*Xho*I of *bep3*, containing the 3′ UTR, is able to bind a protein of 54 kDa (LP54) [14]. Moreover, fragments containing the 3′UTRs of both *bep1* and *bep4*, transcribed in vitro, recognize the same protein [14]. To test the ability of different parts of the 3′UTR of *bep3* to bind to the LP54 we generated DNA constructs in which about 50, 100, 190 or 230 nucleotides starting from the *Acc*I site to the *Xho*I site of the subclone *Acc*I-*Xho*I of *bep3* [10] were deleted by Bal31 treatment (Fig. 2). These constructs were utilized for transcribing in vitro RNAs that were employed as probes in Northwestern binding experiments. Cytoplasmic proteins isolated from *P. lividus* eggs were electrophoretically separated and the Western blots were incubated with labeled transcripts of different sizes.

Fig. 3 shows the results obtained. When transcripts of about 250, 200 and 125 nt were utilized in binding assays the band of 54 kDa was detected whereas when the 80-nt transcript was utilized no reaction was observed. The results indicate that the 3′UTR region alone (about 200 bp) efficiently binds the 54 kDa protein (Fig. 3, lane 3). In particular, the partial structure (about 125 bp) of only stems IV and V obtained after the deletion of about 190 bp still binds the protein, though less efficiently (Fig. 3, lane 4). After deletion of about 230 bp, the remaining structure (about 80 bp) (Fig. 1A) that is part of stem V, shows no binding activity (Fig. 1B, a).

These observations suggest that two stems and loops represent the minimal RNA structure able to bind the protein in *bep3*, *bep1* and *bep4*.

3.3. Microinjection of 3′UTR of bep3 in sea urchin eggs

In order to obtain more direct evidence that the 3′UTR is involved in the anchoring of *bep3* mRNA to the animal cortex region, we microinjected some DIG-conjugate transcripts of the *Acc*I-*Xho*I subclone into unfertilized eggs. After microinjection the eggs were fertilized, in order to cause the transfer of the *bep* RNAs to the cortical region [10]. Detection of 3′UTR was obtained by immunoassay using an antibody alkaline phosphatase conjugate that can react only with the molecules containing UTP-DIG conjugate. As shown in Fig. 4, the injected 3′UTR became localized close to one of the egg

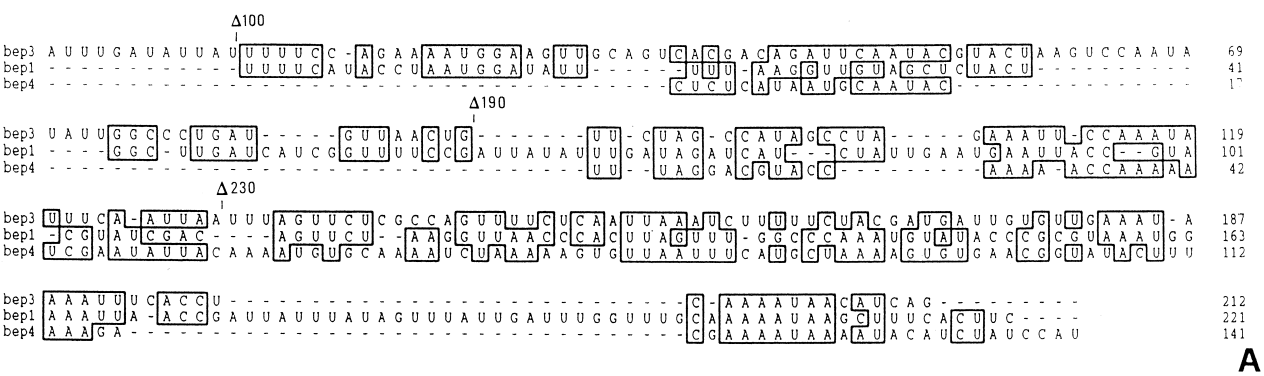

Fig. 1. A: *bep3, bep1, bep4* 3′UTR multialignment. The first nucleotide of each sequence is the first downstream of the stop codon. The boxes highlight nucleotide similarity present in the structures at homologous positions. Δn indicates the point of deletion. B: *bep3* (a), *bep1* (b) and *bep4* (c) 3′UTR predicted secondary structures. Each stem is indicated by Roman numbers. Arrows mark what is left of the *bep3* structure after the deletion of 100, 190 and 230 bases.

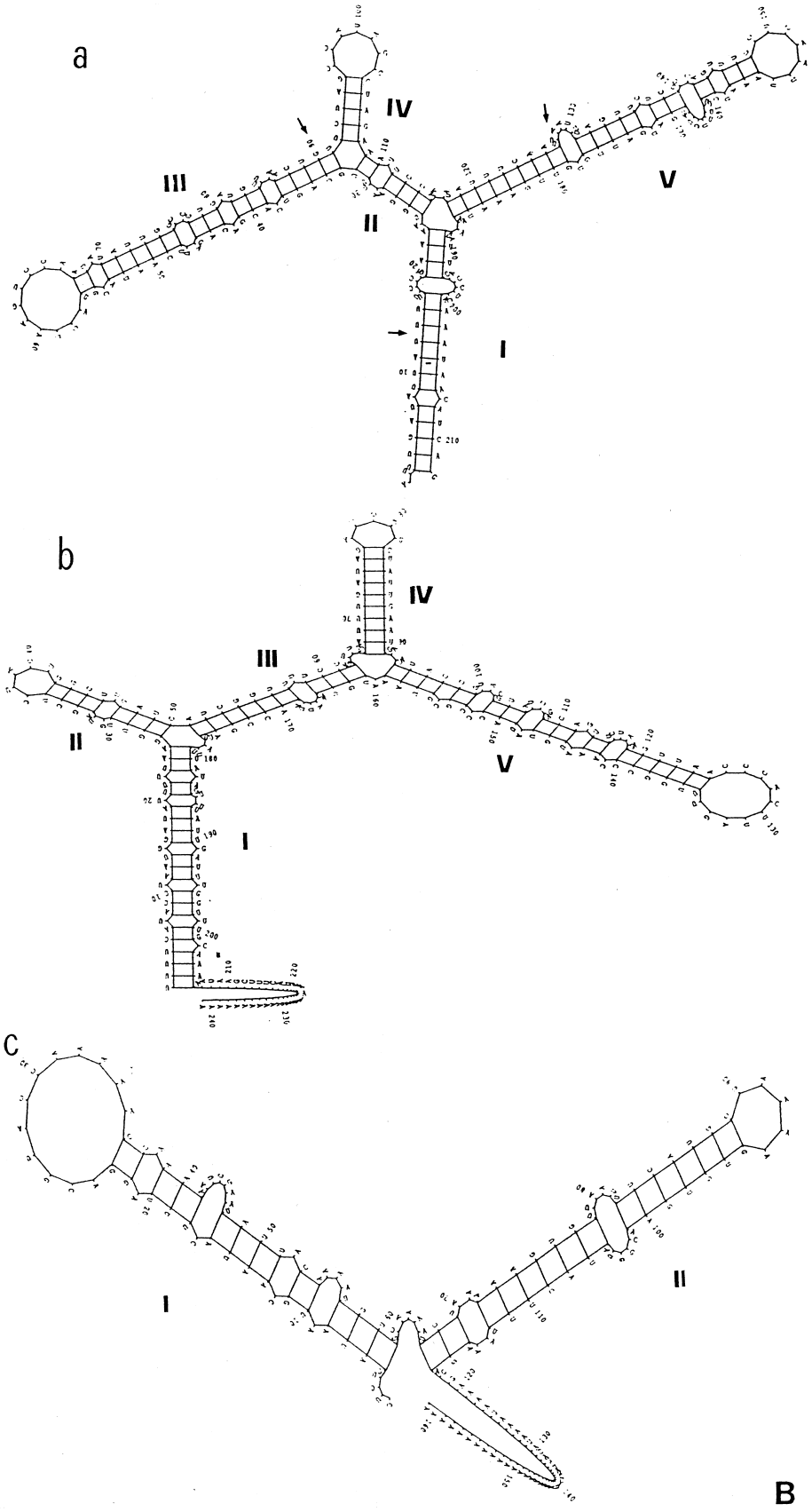

Fig. 1. (*continued*)

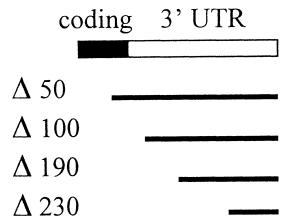

Fig. 2. Deletion in the 3′UTR of *bep3* transcript. Δn indicates the deleted nucleotides of the *Acc*I-*Xho*I *bep3* fragment.

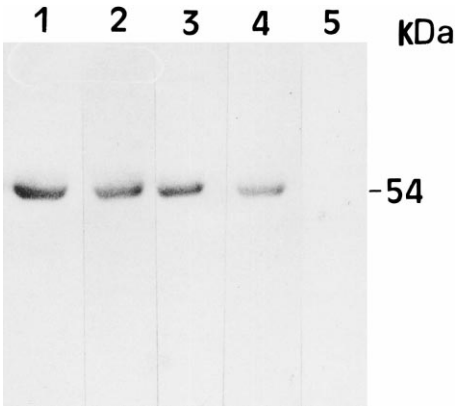

Fig. 3. Northwestern analysis of *bep3* RNA. Western blot of proteins isolated from *P. lividus* eggs, incubated with [^{32}P], *bep3* RNA about 300 nt (1) (control), 250 nt (2), 200 nt (3), 125 nt (4), and 80 nt (5). The molecular weight of the detected bands is indicated.

poles, which, by the presence of the pronucleus, has been shown to be the animal one for the endogenous *bep* RNAs [13]. This result shows that the 3′UTR of *bep3* is able to be transported and anchored to one of the egg poles like the entire *bep* RNA. This indicates that it is the 3′UTR that directs and anchors the *bep3* RNA as previously suggested [14].

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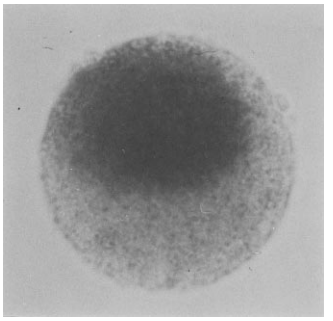

Fig. 4. Localization of exogenous in vitro synthesized 3′UTR of *bep3* injected into unfertilized eggs. Unfertilized eggs injected were fertilized and fixed. The presence of 3′UTR was detected by anti-DIG-AP.

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