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RNA–RNA interaction is required for the formation of specific *bicoid* mRNA 3′ UTR–STAUFEN ribonucleoprotein particles

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The formation of the anterior pattern of the *Drosophila* embryo is dependent on the localization of the mRNA of the morphogen Bicoid (*bcd*) to the anterior pole of the egg cell. Staufen protein (STAU) is required in a late step of the localization to anchor the *bcd* mRNA in the anterior cytoplasm. We have shown previously that endogenous STAU associates specifically with injected *bcd* mRNA 3′-untranslated region (UTR), resulting in the formation of characteristic RNA–protein particles that are transported along microtubules of the mitotic spindles in a directed manner. The regions recognized by STAU in this *in vivo* assay are predicted to form three stem–loop structures involving large double-stranded stretches. Here, we show that the STAU interaction requires a double-stranded conformation of the stems within the RNA localization signal. In addition, base pairing between two single-stranded loops plays a major role in particle formation. This loop–loop interaction is intermolecular, not intramolecular; thus dimers or multimers of the RNA localization signal must be associated with STAU in these particles. The *bcd* mRNA 3′ UTR can also dimerize *in vitro* in the absence of STAU. Thus, in addition to RNA–protein interactions, RNA–RNA interaction might be involved in the formation of ribonucleoprotein particles for transport and localization.

Keywords: Drosophila/dsRNA/RNA dimerization/RNA localization and transport/RNA–protein interactions

Introduction

In *Drosophila*, two RNAs localized at opposite poles of the egg determine the antero-posterior polarity of the embryo, the *bicoid* (*bcd*) mRNA anteriorly, and *oskar* (*osk*) mRNA posteriorly (St Johnston and Nüsslein-Volhard, 1992). These localized RNAs provide sources of protein gradients that ultimately control the transcription of zygotic target genes in a concentration-dependent manner. Localization of RNA is also observed in other germ cells such as *Xenopus* eggs (reviewed in St Johnston, 1995). Although the subcellular localization of mRNAs is best understood in these systems, it is not limited to germ cells and is probably used as a general mechanism for protein targeting in many polarized cell types (St Johnston, 1995).

The localization of *bcd* mRNA is a multistep process that requires three known genes (Frohnhöfer and Nüsslein-Volhard, 1987; Berleth *et al*., 1988; Stephenson *et al*., 1988; St Johnston *et al*., 1989). Mutations in *exuperantia* and *swallow* disrupt the localization of *bcd* mRNA to the anterior cortex of the oocyte during oogenesis. This premature mislocalization leads to an almost homogeneous distribution (*exuperantia*), or to a shallow gradient (*swallow*) of *bcd* mRNA in the embryo. In embryos from *staufen* females, *bcd* mRNA is distributed in a steep anterior gradient, indicating that STAU is required after egg activation to anchor the *bcd* mRNA, after its release from the cortex, in the anterior cytoplasm of the egg (St Johnston *et al*., 1991). STAU contains five double-stranded (ds) RNA-binding motifs common to a family of dsRNA-binding proteins (Green and Mathews, 1992; St Johnston *et al*., 1992; Gatignol *et al*., 1993; Bass *et al*., 1994; Gibson and Thompson, 1994; Kim *et al*., 1994); a peptide containing one STAU dsRNA-binding domain has been shown to bind dsRNA *in vitro*, albeit without sequence specificity (St Johnston *et al*., 1992). STAU is present in excess throughout the *Drosophila* egg but is associated specifically with *bcd* mRNA at the anterior pole. Endogenous STAU also associates with *bcd* mRNA 3′ UTR when injected into the early embryo, resulting in the formation of characteristic RNA–protein particles. Although these particles remain at the site of injection, they associate with and migrate along astral microtubules during mitosis and thus form bipolar patterns that can be visualized at metaphase with the STAU antibody (Ferrandon *et al*., 1994). These observations led us to propose a simple model in which the binding of STAU to its cognate RNA induces a conformational change in STAU that allows it to associate, either directly or indirectly, with a motor that transports the RNA complex.

The *cis*-acting sequences required for all steps of the localization process have been mapped by transgenic analysis to a 625 nucleotide region in the 3′ UTR of the *bcd* mRNA that is predicted to form a complex secondary structure (Macdonald and Struhl, 1988; Macdonald, 1990; Seeger and Kaufman, 1990; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Macdonald *et al*., 1993). The transgenic approach has been successful in identifying elements involved in the localization pathway. However, as most mutations already disrupt the early steps in the localization pathway, the transgenes are not suitable for the analysis of later events such as the interaction with STAU (Macdonald *et al*., 1993; D.Ferrandon and C.Nüsslein-Volhard, in preparation). To circumvent this problem, we have developed an *in vivo* assay based on the injection of *in vitro* synthesized transcripts into the embryo and monitoring their ability to recruit STAU to form RNA–

protein particles that are transported along microtubules (Ferrandon *et al*., 1994). This assay appears faithfully to reproduce the specificity of the interaction of STAU with endogenous RNA since only *bcd* mRNA 3′ UTR shows a strong recruitment of the protein, while a number of control RNAs fail to do so. Further, *bcd* mRNA 3′ UTR particles are not formed in mutant embryos lacking the STAU protein. Using a linker-scanning strategy, we mapped the regions recognized by STAU within the *bcd* mRNA 3′ UTR. They are predicted to form three non-contiguous stem–loop structures involving long double-stranded regions (Ferrandon *et al*., 1994). Here, we show that STAU binding requires the double-stranded conformation of the stems within the RNA localization signal. In addition, base pairing between two single-stranded loops plays a major role in transport particle formation. We show that this loop–loop interaction is intermolecular, and not intramolecular, indicating that dimers or multimers of the RNA localization signal associate with STAU. Thus, the formation of large nucleoprotein transport granules may be dependent on RNA–RNA interactions as well as RNA–protein interactions.

Results

The secondary structure of the bcd mRNA 3′ UTR is required for its specific association with STAU

The regions within the *bcd* mRNA 3′ UTR recognized by STAU were mapped using a linker-scanning strategy (Ferrandon *et al*., 1994). They are predicted (Zuker, 1989) to form three stem–loop structures involving large double-stranded regions: stem–loop III, and the distal parts of stem–loops IV (IVc) and V (Vb) (Figure 1). The same structure is predicted for the *bcd* mRNA 3′ UTR of other species of *Drosophila* (Macdonald, 1990; Seeger and Kaufman, 1990). However, the sequence divergence between those species is relatively low; as a result, the phylogenic approach yields too few compensatory base changes to demonstrate convincingly the base pairing structure (data not shown). To test the relevance of the predicted secondary structure to STAU binding, we designed sets of compensatory mutations on both strands of putative helices. We injected the corresponding RNAs into the early embryo and monitored the association with STAU. The sequences of the mutants are presented in Figure 1 and a typical result is shown in Figure 2a. Single-strand mutations DF503 or DF504 in stem III are each predicted to disrupt base pairing in this region; either of these two mutations prevents the formation of STAU-containing particles by the corresponding injected RNA (Figure 2b and c). The introduction of both mutations into the same transcript results in an RNA in which the base pairs in stem IIIb can still form, but with an altered primary sequence. The injection of this double mutant RNA leads to formation of STAU-containing granules (Figure 2d) (Table I). Thus, the double-stranded helix of stem IIIb is required for the interaction between *bcd* mRNA 3′ UTR and STAU, but the primary sequence is not important. Similar results were obtained with almost all sets of compensatory mutations within predicted helical stems (Table I). In the case of loop IVc, the double-strand mutation DF508 does not restore the ability to interact with STAU, suggesting a sequence-specific interaction of this structure with STAU or with a cofactor. Alternatively, the secondary structure prediction of stem–loop IVc, or that of the double mutant, may be incorrect. In summary, in five out of six cases tested, the preservation of the base complementarity is sufficient for specific association with STAU, despite the substantial changes in the primary sequence of the RNA. This suggests that STAU, containing five RNA-binding motifs, directly binds to several double-stranded regions within the *bcd* mRNA 3′ UTR and that the double-stranded conformation of the RNA within the stem–loops is essential for this binding.

Loop–loop interactions between two bcd mRNA molecules are required for bcd mRNA 3′ UTR–STAU particle formation

Six conserved nucleotides in the distal loop of helix III are perfectly complementary to a side loop of the same helix (Macdonald, 1990) (Figure 1). To examine a possible base pairing between these two loops, we tested a set of compensatory mutations that affect four of the nucleotides in each loop (DF495, DF498). RNA containing the double compensatory mutations (DF496) associates specifically with STAU in the embryo, whereas RNAs containing mutations on either single-stranded region do not (Figure 2e–g). These results suggest that the two loops contact each other through base pairing. One possibility is that this pairing occurs within the molecule, thus forming an element of tertiary structure called a pseudoknot (Westhof and Jaeger, 1992). Attempts to model the putative pseudoknot were unsuccessful because it is not possible to bend stem IIIb sufficiently to allow these two single-stranded loops to base-pair with each other. Moreover, the specific *in vivo* interaction between *bcd* mRNA 3′ UTR and STAU is not affected by the DF497 mutation, which rigidifies this stem further by removing the two asymmetric bulges that form flexible joints. These observations suggest that the two loops cannot base-pair with each other within the same RNA molecule. To test the possibility that base pairing occurs between the loops of different RNA molecules, we injected embryos with a 1:1 mixture of the single loop mutant RNAs, DF495 and DF498. Although neither mutant RNA can interact with STAU on its own, this mixture recruits STAU into particles that are localized (Figure 2h, Table I). Thus, base pairing between the distal and side loops of stem III of two different molecules appears to be required for STAU binding. Indeed, the formation of tetramers or higher order oligomers may account for these results as well as the formation of dimers.

The bcd mRNA 3′ UTR oligomerizes in vitro

To investigate the intermolecular association of the RNA *in vitro*, we incubated *bcd* mRNA 3′ UTR under low or high salt conditions, which respectively hinder or promote base pairing, and separated the RNA by non-denaturing agarose gel electrophoresis (Marquet *et al*., 1991; Tounekti *et al*., 1992). Incubation under low salt conditions results in only one species of the *bcd* mRNA 3′ UTR, whereas under high salt conditions additional bands are observed (Figure 3a). The lowest band most probably corresponds to the monomeric form, whereas the upper bands are likely to correspond to dimers, trimers and tetramers. Similar results are obtained with the 480 nucleotide long DF525 RNA that represents the minimum STAU-binding

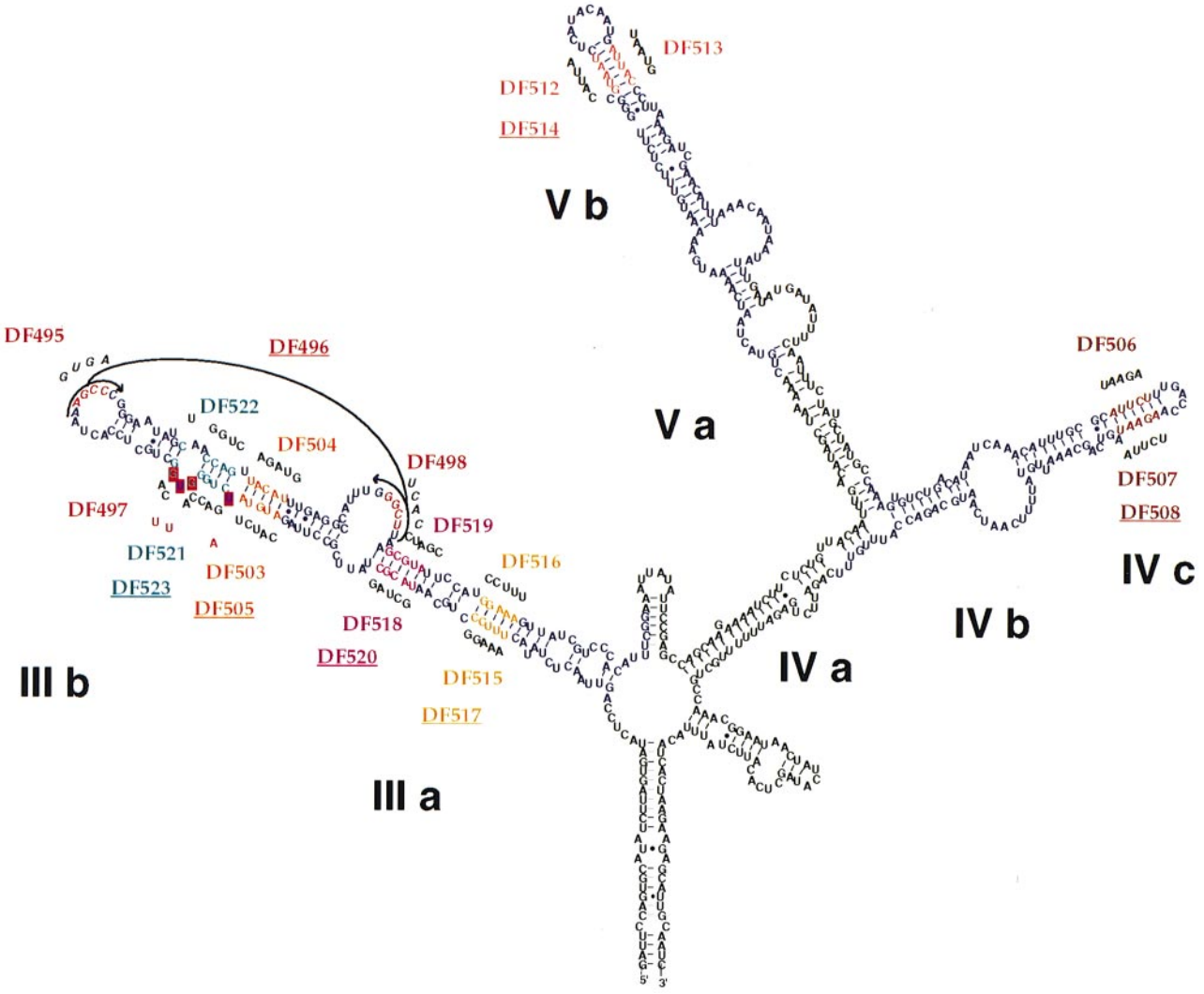

Fig. 1. The proposed secondary structure of the *bcd* mRNA 3′ UTR and the mutations. The core region of the *bcd* mRNA 3′ UTR predicted by the MFOLD program of Zuker (1989) is shown (nucleotides 181–720 of the *bcd* 3′ UTR; G of the UAG stop codon is position 0) (Ferrandon *et al*., 1994). The STAU-binding site is shown in blue (Ferrandon *et al*., 1994). We designed sets of compensatory mutations on both strands of putative helices (designated by Roman numerals) to test directly for such structures. Each set of mutations and its associated name is color coded: the wild-type nucleotides are colored; the corresponding mutations are shown next to them (in black). The name of single-strand mutations is not underlined whereas the name of the corresponding double compensatory mutant is. The nucleotides altered by mutation DF497 are boxed in red; the corresponding mutant nucleotides are also indicated in red. The two connected arrows show the sequence complementarity of six nucleotides of the distal loop of helix III to six nucleotides of its side loop.

site or with a 210 nucleotide long RNA that forms helix III (RNA III) (Figure 3b). In contrast, when a 180 nucleotide long RNA spanning helices Vb and IVc was used, only one band could be detected under both low and high salt conditions, suggesting that this part of the *bcd* mRNA 3′ UTR does not dimerize on its own under our *in vitro* conditions. To test whether the upper bands on the gel do correspond to multimers of the RNA and not to altered conformations of RNA monomers, we mixed equimolar amounts of *bcd* mRNA 3′ UTR and of the short RNA III. Under high salt conditions, this results in a shift of the major band (Figure 3b, lane III+wt), suggesting that this band corresponds to a heterodimer between RNA III and the wild-type RNA. The upper band observed in the mix migrates more slowly than the 3′ UTR dimer band and therefore probably represents heteromultimer forms of these two RNAs. These data indicate that the *bcd* mRNA 3′ UTR can oligomerize *in vitro* and that stem–loop III is involved in the process.

Modeling of the dimer structure reveals a new dimerization motif

The structure of a dimer constituted of two helices III base paired via their side and terminal loops could be successfully assembled and modeled (Figure 4). The loop–loop interaction contains six central Watson–Crick base pairs (A289–C294 to U834–G829) with two tentative non-Watson–Crick pairs at each extremity (A288...A835, and G295...U828), thus forming a mini-helix. The helical domain between the two interacting loops, helix IIIb, is slightly bent at the large asymmetric bulge and its axis is in rough continuity with that of the loop–loop helix. The helical axis of the supporting helix, the end of helix IIIa, is located between those of the two helices IIIb. The dimer

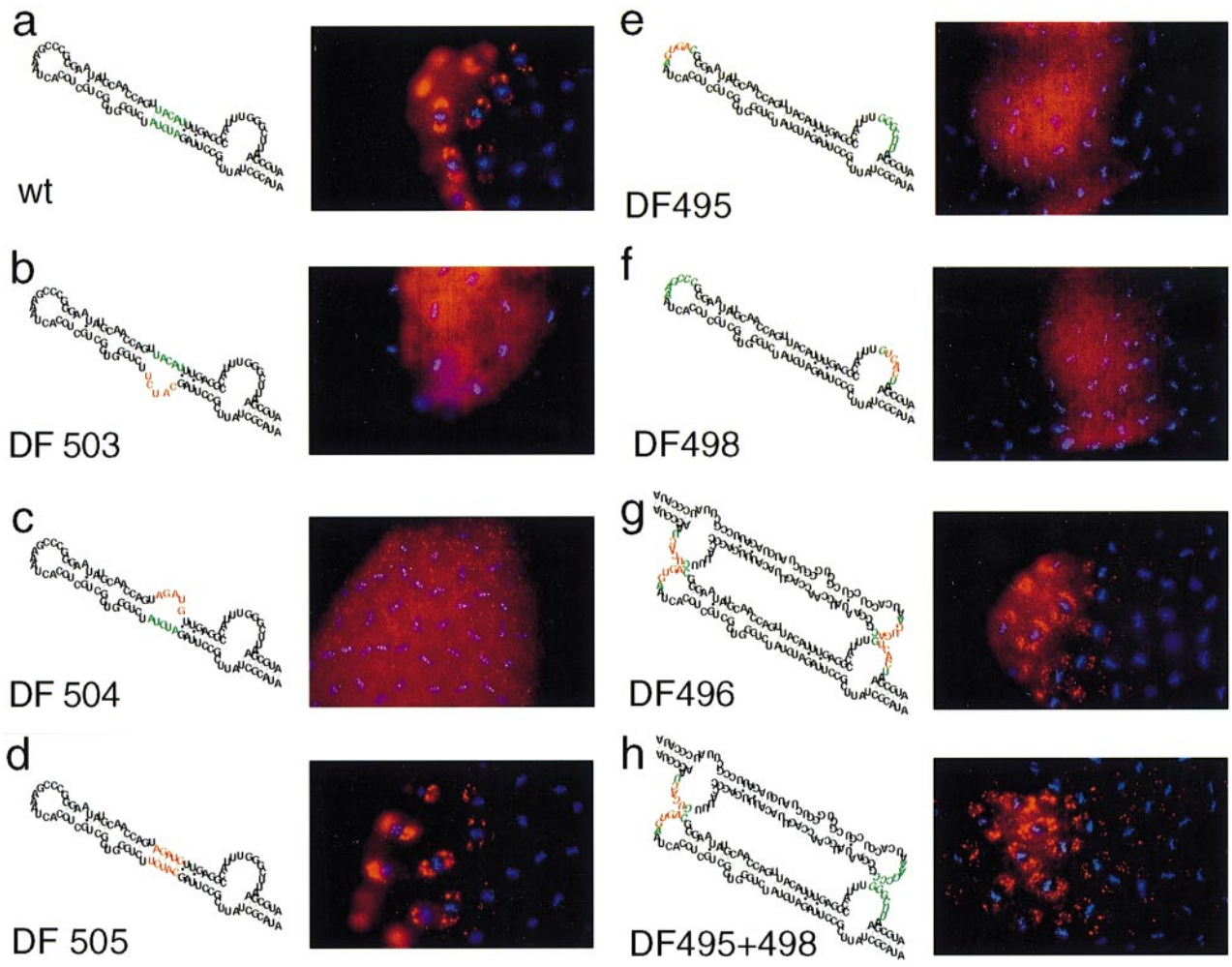

Fig. 2. The specific interaction of the *bcd* mRNA 3′ UTR with STAU *in vivo*. Synthetic *bcd* mRNA 3′ UTR was injected in a small region of cytoplasm of freshly-laid eggs. One hour after injection, the embryos were fixed and stained with a STAU-specific antibody that gives a red signal by indirect immunofluorescence. All RNA batches were encoded before injection and the key was revealed only after scoring of the injected embryos. The position of metaphase plates on the same focal plane is revealed by DAPI (4′,6′-diamidinophenyl indole) staining (blue signal). The left-hand side of each panel represents schematically the structure of helix IIIb of the injected RNA. Nucleotides whose pairing is tested are shown in green. Mutated nucleotides are shown in red. The disruption of helix IIIb induced by mutations DF503 and DF504 is predicted to be much more extensive than schematized here. The STAU–*bcd* mRNA 3′ UTR particles observed with the wild-type (wt) RNA have been shown to associate with astral microtubules (Ferrandon *et al*., 1994). With single-strand mutation RNAs, a non-microtubule-associated signal similar to that produced by the non-specific interaction of STAU with dsRNA was often observed at the injection site, suggesting that STAU recognizes the remaining helices in the structure but is not activated to associate with microtubules (Ferrandon *et al*., 1994). This signal is not easily observed in this figure since the complex remains at the injection point outside of the focal plane of the pictures.

possesses a 2-fold axis of symmetry located half-way on the line connecting the helical axis of helices IIIa. This model suggests that the helices IIIb may be structurally required to orient the loops for efficient base pairing.

Discussion

By testing mutations which change the primary sequence, as well as double mutations restoring base pairing within the stems of the *bcd* mRNA 3′ UTR, we have shown that in most instances the double-stranded conformation and not the primary sequence is crucial for the specific association with the STAU protein. In addition, dimers or oligomers of the RNA must be present in the ribonucleoprotein particles observed *in vivo*. These findings support the notion that STAU, containing five dsRNA-binding motifs, binds directly to the *bcd* mRNA 3′ UTR. We propose that the specificity of this binding is provided by the spatial correspondence between the dsRNA-binding motifs within STAU and the helices found in oligomers of the *bcd* mRNA 3′ UTR. Several proteins contain more than one dsRNA-binding motif, each of which displays little sequence specificity (Green and Mathews, 1992; St Johnston *et al*., 1992; Gatignol *et al*., 1993; Bass *et al*., 1994; Gibson and Thompson, 1994; Kim *et al*., 1994; Bycroft *et al*., 1995). However, in a few instances, sequence-specific interactions with mRNAs have been reported (Polson and Bass, 1994; Davis and Watson, 1996). It is possible that a particular RNA conformation is the basis for recognition in these cases. So far, STAU is the only protein with more than three copies of the dsRNA-binding motif, and this might explain its exquisite structural requirements for RNA recognition.

RNA loop–loop interactions have been reported to play an important role, intermolecularly in the control of DNA replication (Tomizawa, 1993; Marino *et al*., 1995; Predki

Table I. Results of the injection experiments

RNA[a]	Stem–loop[b]	*In vivo* STAU–*bcd* 3′UTR binding[c]
DF503		–
DF504	(IIIb)	–
DF505		+++
DF503 + DF504		–[d]
DF521		–
DF522	(IIIb)	–
DF523		++/+++
DF515		–
DF516	(IIIa)	–
DF517		+++
DF518		–
DF519	(IIIa)	–
DF520		++
DF512		–
DF513	(Vb)	–
DF514		+++
DF506		–
DF507	(IVc)	–
DF508		–/+
DF495		–
DF498		–/+
DF496	(IIIb)	++
DF495 + DF498		+++
DF497	(IIIb)	++

[a]Injected RNAs described in Figure 1. Double compensatory mutants are underlined. For DF503 + DF504 (or DF495 + DF498), a one:one mix of each single-strand mutant was injected.
[b]The number of the stem–loop structure affected by the mutations.
[c]Qualitative scoring of the specific formation of STAU-containing particles observed in ~50–150 injected embryos (Ferrandon *et al*., 1994). Injections where most embryos show a signal similar to that observed in Figure 2a, d and h were scored as +++. A ++ score was given when, in addition to specific particles, a diffuse STAU background staining was detected in most embryos (Figure 2g). For some constructs, a few particles were detected next to nuclei in <10 embryos and were thus given a –/+ score. Finally, injection series where no STAU punctate signal was observed were scored as – (Figure 2b, c, e, and f).
[d]Stem mutations DF503 or DF504 can only be rescued in *cis* (DF505), and not in *trans* (DF503 + DF504), suggesting that the helix is recognized directly by STAU or is required to orient the side and terminal loops so that they can base pair easily.

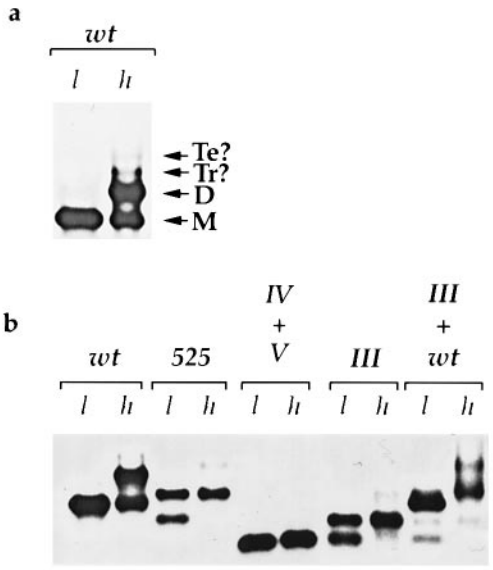

Fig. 3. The *bcd* mRNA 3′ UTR oligomerizes *in vitro*. Synthetic RNAs were incubated under low (l) or high salt conditions (h) which respectively hinder or favor intermolecular association of the RNA (Marquet *et al*., 1991). The two samples were then analyzed side by side by non-denaturing agarose gel electrophoresis. (**a**) wt RNA; several bands are observed under high salt conditions which represent the dimer form (D) (see below) and most likely the trimer (Tr) and tetramer (Te) forms. (**b**) RNA 525 corresponds to the 480 nucleotide long minimum binding site of STAU on the *bcd* mRNA. RNA III corresponds to 210 nucleotides of helix III whereas RNA IV+V contains helices Vb and IVc [DF500 and DF501 respectively (Ferrandon *et al*., 1994)]. Note that dimerization occurs even under low salt conditions for RNAs 525 and III. In the low salt condition lane (l) of the mixing experiment III+wt, one can distinguish successively the following bands from bottom to top: monomer of RNA III, dimer of RNA III, monomer of wt RNA, and finally a very faint band corresponding to a heterodimer between RNA III and wt RNA. This band which is higher than the wt RNA monomer form is seen much more clearly in the high salt condition lane (h). All of the wt monomer RNA form has been converted to this heterodimer band or to a higher heteromultimer band, and not to a wt dimer RNA form, thus demonstrating that the higher bands observed under high salt conditions are indeed dimer or higher multimer forms.

et al., 1995) and in the dimerization of HIV RNA (Paillart *et al*., 1994, 1996; Skripkin *et al*., 1994), and intramolecularly in the autocatalytic group I and group II introns (Michel and Ferat, 1995; Jaeger *et al*., 1996). In our model, helix III appears to be the main element involved in the intermolecular association. However, other parts of the RNA may be required to stabilize the intermolecular RNA interaction. Our data indicate that the *bcd* mRNA 3′ UTR under high salt conditions can form dimers and higher order oligomers even in the absence of STAU, and that helix III is also involved in dimer formation *in vitro*. Modeling studies show that trimers or tetramers can be formed easily by base pairing between the two helix III loops. However, for the macroscopic particles that are observed *in vivo*, the interaction of the RNA with STAU is required. One possibility is that STAU functions as a linker between RNA dimers or short oligomers, either through its five dsRNA-binding motifs or by dimerization itself. Alternatively, STAU could serve to stabilize the RNA–RNA interactions, allowing the formation of multimer chains.

STAU is also required to localize *osk* mRNA to the posterior pole of the oocyte (Ephrussi *et al*., 1991; Kim-Ha *et al*., 1991; St Johnston *et al*., 1991). *osk* mRNA 3′ UTR does not show any homology to the *bcd* mRNA 3′ UTR (Kim-Ha *et al*., 1993). In the case of *osk*, large particles, the polar granules, are formed at the posterior pole of the oocyte that contain, in addition to STAU, a number of identified proteins as well as RNAs (St Johnston, 1993). In contrast to *bcd* mRNA, the interaction of STAU with *osk* mRNA takes place only during oogenesis; in our injection assay, *osk* mRNA 3′ UTR does not associate with STAU (Ferrandon *et al*., 1994). This suggests that other proteins not present in the egg cytoplasm are required for the association of *osk* mRNA with STAU. It will be interesting to see whether the *osk* mRNA 3′ UTR also multimerizes upon association with STAU.

Nucleoprotein particles have been observed in many instances of mRNA transport, both at the optical and electron microscopy level, suggesting that particle formation may play an important role in packaging the RNA for efficient localization (Ainger *et al*., 1993; Trembleau *et al*., 1994, 1995; Forristal *et al*., 1995; Racca *et al*., 1997). The large particles of *bcd* mRNA 3′ UTR and

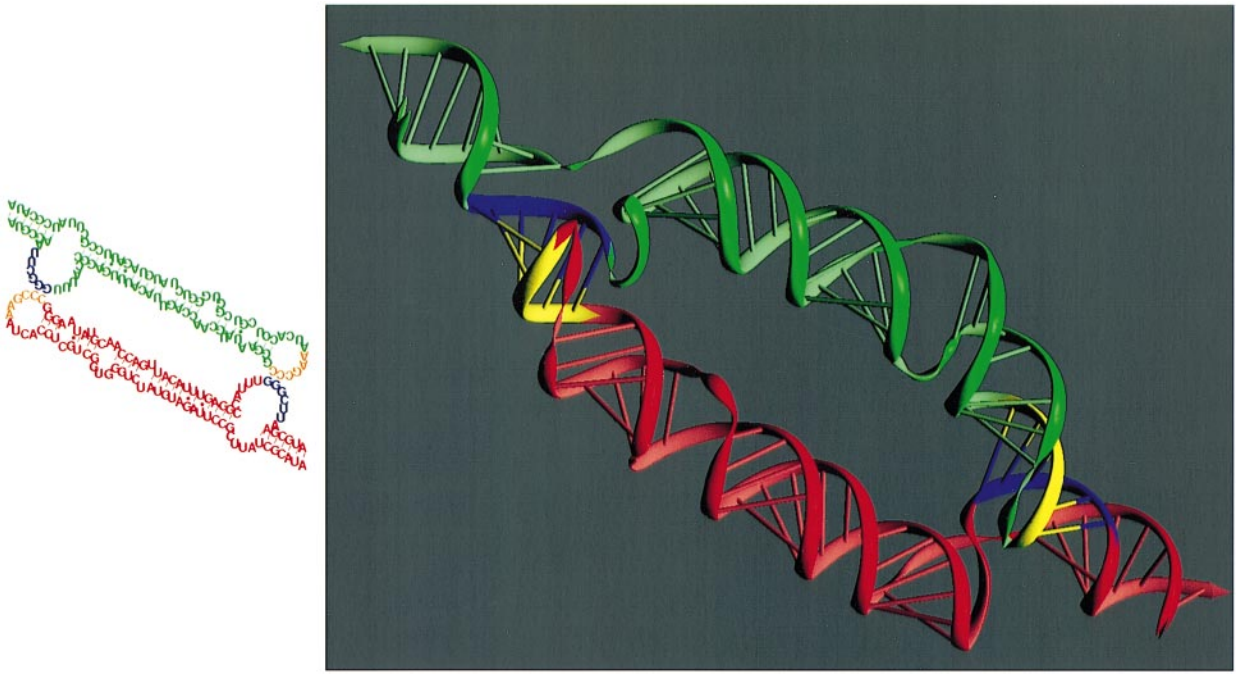

Fig. 4. Model of the dimer interaction between two helices III. Right-hand panel: view of a modeled 3D dimer of hairpin IIIb and the distal part of helix IIIa interacting via loop–loop interactions (one molecule in green, the other in red). The apical loops of both molecules are in yellow and the complementary side loops in dark blue as shown on the 2D model in the left-hand panel. Helix IIIb is 25 bp long (with two mismatches) and, despite the presence of two asymmetric bulges which can constitute flexible joints, it is not possible to bend helix IIIb sufficiently so that the two side loops would base-pair with each other while maintaining the rest of the secondary structure with a correct geometry and stereochemistry of the RNA. This and the results obtained with the injection of DF497 that rigidifies this stem (Figure 1, Table I) make it unlikely that the wild-type RNA forms a pseudoknot. In addition to the dimer, trimer and tetramer models can also be envisaged.

STAU that are detected in the injected embryos are not observed with the endogenous RNA or with injected full-length transcripts. However, smaller particles are observed with full-length transcripts when the cell cycle lengthens at cycle 14 (Ferrandon *et al*., 1994). These transcripts contain large open reading frames, and it may be steric hindrance caused by translation or their greater size that prevents the formation of larger particles. As these full-length *bcd* mRNA molecules also recruit STAU, migrate to the cortex and eventually induce the formation of small particles, it seems likely that they interact with STAU in the same way as the 3′ UTR alone.

The present results suggest that both RNA–RNA and RNA–protein interactions may provide the basis for the formation of very large ribonucleoprotein particles such as the polar granules of *Drosophila* (50 times the size of a ribosome) (St Johnston, 1993), P-granules of *Caenorhabditis elegans* (Seydoux and Fire, 1994) and germinal granules of *Xenopus laevis* (Kloc *et al*., 1993; Forristal *et al*., 1995; Kloc and Etkin, 1995). In conclusion, through its role in mRNA sorting and transport, RNA quaternary structure might turn out to be fundamental to the generation of cell polarity.

Materials and methods

Plasmids and RNA

The mutations were introduced in the *bcd* mRNA 3′ UTR by a two-step PCR procedure as described (Higuchi *et al*., 1988) using DF400 (Ferrandon *et al*., 1994) as a template and the following external primers for all mutant constructs (*bcd* sequences are in capitals): 5′ sense primer, ataagaatgcggccgCCTGGACGAGAGGCGTGT (the first nucleotide is nucleotide 1 of the *bcd* mRNA 3′ UTR) and 3′ antisense primer, ccggaattcAAGGGACGGAAATATGGG (the last nucleotide is nucleotide 870 of the *bcd* mRNA 3′UTR) as well as the following primers for mutagenesis (mutated nucleotides are shown in bold; only the sense primer is indicated since the antisense primers are exactly complementary to the sense): DF503, TATTCGCCTTAG**CATCT**TCTGGGTGGC; DF504, TGCAACCAGT**AGATG**TTGAGGCCATTTG; DF506, AACATTTGCGC**TAAGA**TTGACCAAGAA; DF507, TTGACCA**TCTTA**AGTCAGCAAATTGTA; DF508, ATTTGCTGACT**TAAGA**TGGTCAA**TCTT**; DF509, TCAAAATGAAAA**TACAA**TTCTCTTGGGCGT; DF510, CCCTTAAAGATCG**TTGTA**TTAAACAATAATA; DF512, TTCTCTTGGGC**CATTA**CTCATACAAATG; DF513, CTCATACAATGT**AATG**CCTTAAAGATC; DF515, CCAGTTAACTCTATAC**AAAGG**CTGCAATACGC; DF516, GCGTATTCCAT**CCTTT**GTTATCGTCCC; DF518, TTTCCCTGCAA**GCTAG**TATTCGCCTTA; DF519, TTTGGGCTTAA**CTAGC**TTCCATGGAAA; DF521, CGCCTTAGATGTAT**GACCATCA**CTGCTCCACTAAAG; DF522, CCGGGAATATGT**T**AA**GGTC**TTACATTTGAG.

Double-mutant DNA fragments were obtained by repeating this procedure using one single-strand mutant as a template and the mutagenic primers of the second single-strand mutation. The only exception was DF508, where a specific primer was designed since the stem–loop is too short. After purification and digestion with *Not*I and *Eco*RI, the PCR bands were cloned into *Not*I–*Eco*RI-cut pBNMB vector (Ferrandon *et al*., 1994). The mutations were confirmed by sequencing. Some mutant constructs contain an additional G→T mutation at nucleotide 211. This mutation alone has no effect in the injection assay (data not shown). RNAs were prepared as previously described (Ferrandon *et al*., 1994).

Injection experiments

Experiments were carried out as described (Ferrandon *et al*., 1994). Pictures were taken with a Zeiss Axiophot microscope. The black and white negatives corresponding to the Cy3 channel (STAU) and the DAPI channel were scanned, assigned false colors, and superimposed upon each other using Adobe Photoshop 3.0.

In vitro dimerization experiments

These experiments were carried out as previously described (Marquet *et al*., 1991). Briefly, RNA dissolved in 8 µl of MilliQ-water (Millipore) was heated for 2 min at 90°C and was chilled on ice. Two µl of the

appropriate buffer were then added. For samples incubated under high salt conditions, the tubes were incubated at 37°C and subsequently were chilled on ice. The samples were loaded on 1.1% non-denaturing agarose gels. Electrophoresis was carried out at room temperature in 1× TBM buffer (90 mM Tris-borate pH 8.3, 0.1 mM $MgCl_2$) at 7 V/cm. Gels were then stained in 0.5 mg/ml ethidium bromide.

Incubations were carried out in buffer containing 50 mM sodium cacodylate pH 7.5, and either 40 mM KCl, 0.1 mM $MgCl_2$ (standard monomer buffer: low salt) or 300 mM KCl, 5 mM $MgCl_2$ (standard dimer buffer: high salt). RNA final concentration was usually 0.6 mg/ml. In mixing experiments, equimolar amounts of each RNA were added to a final concentration of 0.6 mg/ml.

RNA modeling

The structure was refined with NUCLIN-NUCLSQ (Westhof *et al*., 1985) and the drawing made with DRAWNA (Massire *et al*., 1994).

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References

Ainger,K., Avossa,D., Morgan,F., Hill,S., Barry,C., Barbarese,E. and Carson,J. (1993) Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. *J. Cell Biol.*, **123**, 431–441.

Bass,B.L., Hurst,S.R. and Singer,J.D. (1994) Binding properties of newly identified *Xenopus* proteins containing dsRNA-binding motifs. *Curr. Biol.*, **4**, 301–314.

Berleth,T., Burri,M., Thoma,G., Bopp,D., Richstein,S., Frigerio,G., Noll,M. and Nüsslein-Volhard,C. (1988) The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.*, **7**, 1749–1756.

Bycroft,M., Grünert,S., Murzin,A.G., Procter,M. and St Johnston,D. (1995) NMR solution structure of a double-stranded RNA-binding domain from *Drosophila* Staufen protein reveals homology to the N-terminal domain of ribosomal protein S5. *EMBO J.*, **14**, 3563–3571.

Davis,S. and Watson,J.C. (1996) *In vitro* activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3′ untranslated regions of human alpha-tropomyosin. *Proc. Natl Acad. Sci. USA*, **93**, 508–513.

Ephrussi,A. and Lehmann,R. (1992) Induction of germ cell formation by *oskar*. *Nature*, **358**, 387–392.

Ephrussi,A., Dickinson,L.K. and Lehmann,R. (1991) *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell*, **66**, 37–50.

Ferrandon,D., Elphick,L., Nüsslein-Volhard,C. and St Johnston,D. (1994) Staufen protein associates with the 3′ UTR of *bicoid* mRNA to form particles that move in a microtubule-dependent manner. *Cell*, **79**, 1221–1232.

Forristal,C., Pondel,M., Chen,L. and King,M.L. (1995) Patterns of localization and cytoskeletal association of two vegetally localized RNAs, *Vg1* and *Xcat-2*. *Development*, **121**, 201–208.

Frohnhöfer,H.G. and Nüsslein-Volhard,C. (1987) Maternal genes required for the anterior localization of *bicoid* activity in the embryo of *Drosophila*. *Genes Dev.*, **1**, 880–890.

Gatignol,A., Buckler,C. and Jeang,K.T. (1993) Relatedness of an RNA–binding motif in human–immunodeficiency-virus type-1 TAR RNA-binding protein TRBP to human P1/DSI kinase and *Drosophila* Staufen. *Mol. Cell. Biol.*, **13**, 2193–2202.

Gavis,E.R. and Lehmann,R. (1992) Localization of *nanos* RNA controls embryonic polarity. *Cell*, **71**, 310–313.

Gibson,T.J. and Thompson,J.D. (1994) Detection of dsRNA-binding domains in RNA helicase A and *Drosophila* maleless: implications for monomeric RNA helicases. *Nucleic Acids Res.*, **22**, 2552–2556.

Green,S.R. and Mathews,M.B. (1992) Two RNA-binding motifs in the double-stranded RNA-activated protein-kinase, DAI. *Genes Dev.*, **6**, 2478–2490.

Higuchi,R., Krummel,B. and Saiki,R.K. (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.*, **16**, 7351–7364.

Jaeger,L., Michel,F. and Westhof,E. (1996) The structure of group I ribozymes. In Eckstein,F. and Lilley,D.M.J. (eds), *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, pp. 33–51.

Kim,J.L., Wang,Y., Sanford,T., Zeng,Y. and Nishikura,K. (1994) Molecular cloning of cDNA for double-stranded RNA adenosine deaminase, a candidate enzyme for nuclear RNA editing. *Proc. Natl Acad. Sci. USA*, **91**, 11457–11461.

Kim-Ha,J., Smith,J.L. and Macdonald,P.M. (1991) *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell*, **66**, 23–35.

Kim-Ha,J., Webster,P., Smith,J. and Macdonald,P. (1993) Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development*, **119**, 169–178.

Kloc,M. and Etkin,L.D. (1995) Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes. *Development*, **121**, 287–297.

Kloc,M., Spohr,G. and Etkin,L.D. (1993) Translocation of repetitive RNA sequences with the germ plasm in *Xenopus* oocytes. *Science*, **262**, 1712–1714.

Macdonald,P.M. (1990) *bicoid* mRNA localization signal: phylogenetic conservation of function and RNA secondary structure. *Development*, **110**, 161–71.

Macdonald,P.M. and Struhl,G. (1988) *Cis*-acting sequences responsible for anterior localization of *bicoid* mRNA in *Drosophila* embryos. *Nature*, **336**, 595–598.

Macdonald,P.M., Kerr,K., Smith,J.L. and Leask,A. (1993) RNA regulatory element Ble1 directs the early steps of *bicoid* messenger-RNA localization. *Development*, **118**, 1233–1243.

Marino,J.P., Gregorian,R.S.,Jr, Csankovszki,G. and Crothers,D.M. (1995) Bent helix formation between RNA hairpins with complementary loops. *Science*, **268**, 1448–1454.

Marquet,R., Baudin,F., Gabus,C., Darlix,J.-L., Mougel,M., Ehresmann,C. and Ehresmann,B. (1991) Dimerization of human immunodeficiency virus (type 1) RNA: stimulation by cations and possible mechanism. *Nucleic Acids Res.*, **19**, 2349–2357.

Massire,C., Gaspin,C. and Westhof,E. (1994) DRAWNA: a program for drawing schematic views of nucleic acids. *J. Mol. Graphics*, **12**, 201–206.

Michel,F. and Ferat,J.L. (1995) Structure and activities of group II introns. *Annu. Rev. Biochem.*, **64**, 435–461.

Paillart,J.-C., Marquet,R., Skripkin,E., Ehresmann,B. and Ehresmann,C. (1994) Mutational analysis of the bipartite dimer linkage structure of human immunodeficiency virus type 1 genomic RNA. *J. Biol. Chem.*, **269**, 27486–27493.

Paillart,J.-C., Skripkin,E., Ehresmann,B., Ehresmann,C. and Marquet,R. (1996) A loop–loop 'kissing' complex is the essential part of the dimer linkage of genomic HIV-1 RNA. *Proc. Natl Acad. USA*, **93**, 5572–5577.

Polson,A.G. and Bass,B.L. (1994) Preferential selection of adenosines for modification by double-stranded RNA adenosine deaminase. *EMBO J.*, **13**, 5701–5711.

Predki,P.F., Nayak,M., Gottlieb,M.B.C. and Regan,L. (1995) Dissecting RNA–protein interactions: RNA–RNA recognition by Rop. *Cell*, **80**, 41–50.

Racca,C., Gardiol,A. and Triller,A. (1997) Dendritic post-synaptic localization of alpha-glycin receptor subunit mRNAs. *J. Neurosci.*, **17**, in press.

Seeger,M.A. and Kaufman,T.C. (1990) Molecular analysis of the *bicoid* gene from *Drosophila pseudoobscura*: identification of conserved domains within coding and noncoding regions of the *bicoid* mRNA. *EMBO J.*, **9**, 2977–2987.

Seydoux,G. and Fire,A. (1994) Soma–germline asymmetry in the distributions of embryonic RNAs in *Caenorhabditis elegans*. *Development*, **120**, 2823–2834.

Skripkin,E., Paillart,J.-C., Marquet,R., Ehresmann,B. and Ehresmann,C. (1994) Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization *in vitro*. *Proc. Natl Acad. Sci. USA*, **91**, 4945–4949.

St Johnston,D. (1993) Pole plasm and the posterior group of genes. In Bate,M. and Martinez-Arias,A. (eds), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. I, pp. 325–363.

St Johnston,D. (1995) The intracellular localization of mRNAs. *Cell*, **81**, 161–170.

St Johnston,D. and Nüsslein-Volhard,C. (1992) The origin of pattern and polarity in the *Drosophila* embryo. *Cell*, **68**, 201–219.

St Johnston,D., Driever,W., Berleth,T., Richstein,S. and Nüsslein-Volhard,C. (1989) Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Development Suppl.*, **107**, 13–19.

St Johnston,D., Beuchle,D. and Nüsslein-Volhard,C. (1991) *Staufen*, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell*, **66**, 51–63.

St Johnston,D., Brown,N.H., Gall,J.G. and Jantsch,M. (1992) A conserved double-stranded RNA-binding domain. *Proc. Natl Acad. Sci. USA*, **89**, 10979–10983.

Stephenson,E.C., Chao,Y.C. and Fackenthal,J.D. (1988) Molecular analysis of the *swallow* gene of *Drosophila melanogaster*. *Genes Dev.*, **2**, 1655–1665.

Tomizawa,J.-I. (1993) Evolution of functional structures of RNA. In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 419–445.

Tounekti,N., Mougel,M., Roy,C., Marquet,C., Darlix,J.-L., Paoletti,J., Ehresmann,B. and Ehresmann,C. (1992) Effect of dimerization on the conformation of the encapsidation Psi domain of Moloney murine leukemia virus RNA. *J. Mol. Biol.*, **223**, 205–220.

Trembleau,A., Morales,M. and Bloom,F.E. (1994) Aggregation of vasopressin mRNA in a subset of axonal swellings of the median eminence and posterior pituitary: light and electron microscopy evidence. *J. Neurosci.*, **14**, 39–53.

Trembleau,A., Melia,K.R. and Bloom,F.E. (1995) BC1 RNA and vasopressin mRNA in rat neurohypophysis: axonal compartmentalization and differential regulation during dehydration and rehydration. *Eur. J. Neurosci.*, **7**, 2249–2260.

Westhof,E. and Jaeger,L. (1992) RNA pseudoknots. *Curr. Opin. Struct. Biol.*, **2**, 327–333.

Westhof,E., Dumas,P. and Moras,D. (1985) Crystallographic refinement of yeast aspartic acid transfer RNA. *J. Mol. Biol.*, **184**, 119–145.

Zuker,M. (1989) On finding all suboptimal foldings of an RNA molecule. *Science*, **244**, 48–52.

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