Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation

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Drosophila Staufen protein is required for the localization of oskar mRNA to the posterior of the oocyte, the anterior anchoring of bicoid mRNA and the basal localization of prospero mRNA in dividing neuroblasts. The only regions of Staufen that have been conserved throughout animal evolution are five double-stranded (ds)RNA-binding domains (dsRBDs) and a short region within an insertion that splits dsRBD2 into two halves. dsRBDs 1, 3 and 4 bind dsRNA in vitro, but dsRBDs 2 and 5 do not, although dsRBD2 does bind dsRNA when the insertion is removed. Full-length Staufen protein lacking this insertion is able to associate with oskar mRNA and activate its translation, but fails to localize the RNA to the posterior. In contrast, Staufen lacking dsRBD5 localizes oskar mRNA normally, but does not activate its translation. Thus, dsRBD2 is required for the microtubule-dependent localization of osk mRNA, and dsRBD5 for the derepression of oskar mRNA translation, once localized. Since dsRBD5 has been shown to direct the actin-dependent localization of prospero mRNA, distinct domains of Staufen mediate microtubule- and actin-based mRNA transport. Keywords: bicoid mRNA/dsRNA-binding domain/mRNA localization/Staufen/translational control

Introduction

The establishment of cell polarity requires the targeting of specific proteins to the regions of a cell where they are required, and this is often achieved by localizing the mRNAs that encode them (St Johnston, 1995; Bashirullah et al., 1998). In many cases, mRNA localization is thought to be an active process that requires the cytoskeleton. For example, mating type switching in Saccharomyces cerevisiae is restricted to the mother cell by the myosin-dependent transport of ash1 mRNA into the emerging daughter cell, and the directed motility of cultured fibroblasts requires the actin-dependent localization of β-actin mRNA (Kislauskis et al., 1994; Bertrand et al., 1998). Other mRNAs are localized by microtubule-dependent mechanisms, such as Vg1 mRNA, which moves to the vegetal pole of the Xenopus oocyte, and bicoid (bcd) and oskar (osk) mRNAs, which localize to opposite poles of the Drosophila oocyte (Yisraeli et al., 1990; Pokrywka and Stephenson, 1991; Clark et al., 1994). The importance of microtubule-dependent mRNA localization has been most clearly demonstrated in the case of the latter two transcripts, since their positions define the anterior–posterior axis of the embryo. bcd mRNA localizes to the anterior of the egg, and is translated after fertilization to produce a morphogen gradient that patterns the head and thorax of the embryo; the localization of osk mRNA to the posterior of the oocyte directs the assembly of the pole plasm, which contains posterior and germline determinants (Ephrussi and Lehmann, 1992; Driever, 1993).

The cis-acting signals that direct mRNA localization have been mapped in several transcripts, and in a few cases biochemical approaches have led to the identification of RNA-binding proteins that interact with these signals (Bashirullah et al., 1998). However, the best characterized example of an RNA-binding protein required for mRNA localization is Drosophila Staufen protein (Stau), which was identified in a genetic screen (Schüpbach and Wieschaus, 1986). Subsequent work has shown that Stau plays an essential role in the localization of three different mRNAs during development. It is required for (i) the localization of osk mRNA to the posterior of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991, 1995); (ii) the anchoring of bcd mRNA at the anterior of the egg (St Johnston et al., 1989); and (iii) the basal localization of prospero mRNA during the asymmetric divisions of embryonic neuroblasts (Li et al., 1997; Broadus et al., 1998; Matsuzaki et al., 1998; Schultk et al., 1998; Shen et al., 1998). Although it has not been possible to test whether Stau binds specifically to these mRNAs, it contains five copies of a double-stranded (ds)RNA-binding domain (dsRBD), and the third of these has been shown to bind to dsRNA in vitro (St Johnston et al., 1992). When mutations that abolish the RNA-binding activity of dsRBD3 are incorporated into a full-length Stau transgene, this construct no longer rescues the localization of either osk or bcd mRNAs (Ramos et al., 2000). Thus, the dsRNA-binding activity of dsRBD3 is required for bcd and osk mRNA localization, strongly suggesting that Stau binds these RNAs directly.

During stages 7–9 of Drosophila oogenesis, osk mRNA localizes transiently at the anterior of the oocyte, and then moves to the posterior pole at stage 9 in a microtubule-dependent manner (Ephrussi et al., 1991; Kim-Ha et al., 1991; Clark et al., 1994). In stau null mutants, however, osk mRNA fails to move to the posterior and remains at the anterior. Several lines of evidence indicate that Stau protein associates with osk mRNA to mediate its posterior
localization. (i) Staufen protein co-localizes with osk mRNA at the anterior of the oocyte and moves with the RNA to the posterior (St Johnston et al., 1991). (ii) Staun and osk RNA mislocalize to the same ectopic sites in mutants, such as gurken, which alter the polarity of the oocyte (González-Reyes et al., 1995; Roth et al., 1995). (iii) The
posterior localization of Stau depends on osk mRNA (Ferrandon et al., 1994). In females carrying extra copies of an osk transgene, the increased quantity of osk mRNA produced induces a corresponding increase in the amount of Stau that localizes to the posterior pole. Thus, Stau is present in excess, and only the protein that is associated with osk RNA localizes to the posterior.

Translation of unlocalized osk mRNA is repressed by the binding of Bruno protein to Bruno-response elements (BRE) in the 3’UTR (Kim-Ha et al., 1995; Gunkel et al., 1998). An osk transgene lacking the BRE (oskBRE–) is therefore translated prior to its localization, leading to the production of ectopic Osk, which causes a range of patterning defects in the resulting embryos. Low levels of ectopic Osk result in a loss of head and thoracic segments, while higher levels induce the formation of bicaudal embryos, with abdomens at both ends. Although the translation of oskBRE– mRNA no longer depends on its localization, it still requires Stau protein, since the bicaudal phenotypes are suppressed in a stau null mutant background. Thus, Stau plays a role in the translation of oskBRE– mRNA that is independent of its role in posterior localization, suggesting that it may also be involved in the translational regulation of wild-type osk mRNA.

Finally, Stau has also been implicated in the anchoring of osk mRNA at the posterior. When a temperature-sensitive stau allele is kept under semi-restrictive conditions, Stau and osk mRNA localize to the posterior of the oocyte at stage 10, but are not maintained there in the embryo (St Johnston et al., 1991; Rongo et al., 1995).

Although Stau is not involved in the initial anterior localization of bcd mRNA, it is required to anchor the mRNA during the final stages of oogenesis (St Johnston et al., 1989). bcd RNA is normally released from the cortex at some time between stage 12 of oogenesis and egg deposition, and remains tightly localized in a spherical region of cytoplasm at the anterior of the egg. In stau mutant eggs, however, the RNA forms a shallow anterior–posterior gradient, and the resulting embryos have head defects because there is insufficient Bicoid protein at the very anterior of the embryo. This function of Stau shows several parallels to its role in Prospero localization of both bcd and osk mRNAs, but at two different stages of development. Furthermore, in each case the localization of Stau requires its interaction with the appropriate RNA, suggesting that Stau–RNA complexes are the substrate for localization.

More recently, Stau has been shown to mediate the localization of prospero mRNA during the asymmetric divisions of embryonic neuroblasts (Li et al., 1997; Broodus et al., 1998; Fuerstenberg et al., 1998; Matsuzaki et al., 1998; Schultd et al., 1998; Shen et al., 1998). In contrast to the localization of bcd and osk mRNAs, the localization of prospero mRNA–Stau complexes is disrupted by actin-destabilizing drugs, but not by microtubule-depolymerizing drugs (Broodus and Doe, 1997).

The discovery that Stau can mediate both microtubule- and actin-dependent mRNA localization raises the question of how different Stau–mRNA complexes are coupled to distinct transport pathways. It has previously been shown that Miranda protein binds to the dsRBD5 of Stau to direct the basal localization of prospero mRNA (Fuerstenberg et al., 1998; Schuldt et al., 1998; Shen et al., 1998). However, it remains unclear how Stau links osk and bcd mRNAs to the microtubule-based transport machinery, or how the activation of osk mRNA translation at the posterior is achieved. In this paper, we address this question by analysing the domains of Stau to determine which regions of the protein are required for these functions.

Results

Conservation of Stau throughout the animal kingdom

Since the functional domains of a protein can often be identified from their conservation during evolution, we cloned and sequenced homologues of stau from two other insect species, Drosophila virilis (Dvstau) and Musca domestica (Mdstau), which diverged from Drosophila melanogaster over 60 and 100 million years ago, respectively. In addition, Wickham et al. (1999) and Marión et al. (1999) have recently reported Stau homologues in Caenorhabditis elegans (Cstau), mouse (Mmstau) and human (Hstau), and we also identified these on the basis of their homology to the insect genes and sequenced them in their entirety. The predicted amino acid sequences of the invertebrate homologues include five dsRBDs, corresponding to dsRBDs 1–5 of D.melanogaster Stau. In contrast, the human and mouse homologues include only four domains, which are most closely related to dsRBDs 2, 3, 4 and 5 of the invertebrate Stau (Figure 1D).

Despite the absence of a dsRBD1 equivalent in the mouse and human sequences, we believe that they represent vertebrate homologues of Stau because of the high degree of similarity to DmStau within the remaining four domains (Figure 1A). A ClustalW analysis of dsRBDs from many proteins reveals that apart from one minor exception for dsRBD5, a given Stau domain is most similar to the equivalent domain in each of the other Stau homologues (Figure 1D). This suggests that evolution is not acting simply to maintain similarity to a dsRBD consensus sequence, but rather that each domain has unique features that have been conserved during evolution. It is particularly notable that in all six homologues, dsRBD2 is split into two parts by up to 118 aa of non-dsRBD sequence. While dsRBDs have been identified in many proteins, the distinctive split in dsRBD2 has only been observed in these six Stau homologues.

Analysis of an alignment of the Stau homologues reveals that the only portions of the protein to have been conserved during evolution are the five dsRBDs (Figure 1B). For example, the M.domestica and D.melanogaster proteins show an average of 67% amino acid identity within the dsRBDs, but <15% in the rest of the protein. dsRBD2 and dsRBD5 were originally described as ‘half domains’ showing similarity to the dsRBD consensus only over the C-terminal portion of the domain (St Johnston et al., 1992). However, the conservation extends over a region corresponding to the length of a whole domain, and these
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Fig. 2. The dsRBDs are the only conserved regions of Stau required for osk mRNA localization. (A) The localization of Stau protein (i and ii) and osk mRNA (iii and iv) in stage 9 and 10 oocytes from \textit{stauf}\textsuperscript{D3} T\textsuperscript{[Staufull]/\textit{stauf}\textsuperscript{D3}} females. Full-length Stau protein expressed from the transgene localizes normally to the posterior of the oocyte, and rescues the osk mRNA posterior localization defect of a \textit{staufen} null mutation. (B) The localization of Stau protein (i and ii) and osk mRNA (iii and iv) in stage 9 and 10 oocytes from \textit{stauf}\textsuperscript{D3} T\textsuperscript{[Stau\textsuperscript{AN}/\textit{stauf}\textsuperscript{D3}} females. Stau protein lacking the non-conserved N-terminal 282 aa also localizes normally and rescues osk mRNA localization and anchoring. (C) (i) In wild-type egg chambers, osk mRNA shows a transient localization to the anterior of the oocyte during stage 9. (ii) In \textit{stauf}\textsuperscript{D3} T\textsuperscript{[StaudoM/\textit{stauf}\textsuperscript{D3}} egg chambers, osk mRNA fails to accumulate at the anterior margin at stage 9, and localizes instead to the centre of the oocyte. (iii) However, the mRNA shows a normal localization at the posterior pole by stage 10. (iv) In \textit{stauf}\textsuperscript{D3} egg chambers, all osk mRNA remains anchored at the anterior of the oocyte. (v) osk mRNA shows a transient localization to a point in the centre of the oocyte, when StaudoMd is expressed in the presence of wild-type \textit{Drosophila} Stau protein. (D) A diagram showing the structure of the Stau proteins encoded by the Staufull, Stau\textsuperscript{AN}, Stau\textsuperscript{DmMd}, Stau\textsuperscript{Aloop2} and Stau\textsuperscript{AdSRBD5} transgenes. The boxes indicate the positions of the dsRBDs. The short leader peptide containing the myc epitope tag is labelled in blue, \textit{Drosophila} sequences in green and \textit{M.domestica} sequences in red or pink.

domains should therefore be considered as complete, albeit divergent, dsRBDs, in agreement with the results of Gibson and Thompson (1994). The only other obvious homology between these proteins is a short region within the insertion in the middle of dsRBD2 that is rich in proline and aromatic amino acids (Figure 1B and C). Since the regions of the protein essential for its activity are expected to be conserved during evolution, the dsRBDs and this proline-rich region are likely to mediate all of the functions of Staup, including its ability to bind both mRNA and the factors that localize Staup–mRNA complexes.

To determine whether the dsRBDs are indeed the only part of Staup necessary for its function, we generated a transgene in which the large non-conserved N-terminal region of DmStau was deleted, and crossed this construct into a \textit{stauf} null mutant background (\textit{stauf}\textsuperscript{D3}). Like the full-length protein expressed from the same vector (Staufull), Stau\textsuperscript{AN} localizes normally to the posterior of the oocyte, and completely rescues the posterior localization and anchoring of osk mRNA (Figure 2A and B). Furthermore, the eggs laid by Stau\textsuperscript{AN} females show a wild-type localization of bcd mRNA at the anterior pole (data not shown). This rescue of the maternal function of \textit{stauf} is also reflected in the phenotype of the embryos produced by Stau\textsuperscript{AN} females. Whereas the embryos laid by \textit{stauf}\textsuperscript{D3} mutant females die with head defects and no abdomen, almost all of the progeny of Stau\textsuperscript{AN} females hatch into larvae, and have normal heads and almost wild-type abdominal segmentation (Table I). Furthermore, a similar proportion of the adult offspring of Stau\textsuperscript{AN} females had gametic ovaries (85%) compared with those of females carrying Staufull (88%), indicating that this construct leads to the production of the high levels of Osk activity that are necessary to specify the germline (Table I). Thus, the portion of Stau that includes the dsRBDs is able to mediate all of Stau’s functions during oogenesis, including localization of both osk and bcd mRNAs, activation of
osk translation and maintenance of pole plasm at the posterior.

As a more stringent test of whether the functional domains of Stau have been conserved during evolution, we generated transgenic lines in which the dsRBD-containing region of DmStau is replaced with the corresponding region from *M. domestica* (Stau^DmMd^), and found that this transgene rescues all the phenotypes of a *stau* null mutation at least as well as the full-length Stau construct (Figure 2C; Table I). Since the only regions that are conserved between DmStau and MdStau are the five dsRBDs and the short sequence in the insertion in dsRBD2, it is likely that the most important functional domains of the protein reside in these regions.

Although the localization of osk mRNA to the posterior of the oocyte during stage 9 appears normal in *stau*^D3^ females carrying Stau^DmMd^, osk mRNA localizes through an atypical intermediate stage. In wild-type flies, osk mRNA shows a transient association with the anterior pole of the oocyte before moving to the posterior, whereas all of the mRNA remains at the anterior in *stau*^D3^ homozygous females (Figure 2C, i and iv). In Stau^DmMd^ ovaries, however, osk mRNA does not accumulate at the anterior of the oocyte, and instead forms a ‘blob’ in the middle of the oocyte, which disperses as localization proceeds (Figure 2C, ii and iii). Furthermore, this effect of Stau^DmMd^ is dominant: in the absence of endogenous *D. melanogaster* Stau this ‘blob’ is diffuse, but in a wild-type background it forms a much sharper, well defined spot (Figure 2C, v). The nature of the cytoplasmic blob is unclear, but it is intriguing that *D. virilis* osk mRNA is localized through a similar intermediate when introduced into *D. melanogaster* (Webster et al., 1994).

dsRBD2 and dsRBD5 do not bind dsRNA

The discovery that the dsRBDs of Stau are the only conserved regions of the protein that are required for its function raises the question of whether all of these domains bind to dsRNA, and we therefore examined the ability of the five dsRBDs to bind to dsRNA on Northwestern blots (Figure 3A). As previously reported, dsRBD3 binds strongly to dsRNA, whereas a control domain in which 5 aa that contact the RNA have been mutated does not (Ramos et al., 2000). dsRBDs 1 and 4 also bind dsRNA, irrespective of its sequence, although this binding is weaker than that observed with dsRBD3. In contrast, dsRBD2 and dsRBD5 do not bind to any of the dsRNAs tested in this assay.

NMR and mutational analysis of Stau dsRBD3–dsRNA complexes have revealed that the dsRBD binds RNA through conserved amino acids that cluster on one face of the domain (Figure 3C) (Ramos et al., 2000). The corresponding positions in dsRBDs 1, 4 and 5 can be identified both by sequence alignment and by ‘threading’ their sequences onto the structure of dsRBD3. While dsRBDs 1 and 4 contain identical or similar conserved amino acids to dsRBD3, the amino acids on this face of dsRBD5 are much less well conserved, and are of a different type from those found in the other domains (Figure 3C). The lack of conservation of the dsRNA-contacting amino acids, coupled with the observed inability of dsRBD5 to bind dsRNA *in vitro* strongly suggest that this domain does not function as a dsRBD *in vivo*. However, the structural amino acids that comprise the hydrophobic core of the domain are highly conserved when compared with other dsRBDs, and amino acids on the other faces of dsRBD5 are also conserved across the different species. It is therefore likely that domain 5 folds into a typical dsRBD structure, but performs a distinct conserved function unrelated to dsRNA binding.

A highly conserved feature of all six Stau homologues is the presence of the large loop interrupting dsRBD2, and this most probably accounts for the inability of this domain to bind dsRNA *in vitro*. The NMR structure of the dsRBD3–dsRNA complex reveals that the dsRNA-binding regions of the domain span one turn of a dsRNA helix, and that their relative positions within the whole domain are crucial for RNA binding (Ramos et al., 2000). The insertion in loop 2 separates the two halves of dsRBD2, and the RNA-binding amino acids are therefore unlikely to have the correct spacing to contact RNA. Although the presence of the insertion in loop 2 makes it impossible to predict the structure of dsRBD2, the sequence of the domain suggests that it could bind dsRNA if it adopted a conformation in which these two halves were juxtaposed. To test this hypothesis, we constructed a version of dsRBD2 in which the extended loop 2 is replaced by the corresponding 8 aa loop of dsRBD3. When examined in the Northwestern assay, this dsRBD2aloop2 binds dsRNA almost as efficiently as dsRBD3 (Figure 3). Thus, dsRBD2 can bind dsRNA when the removal of the large insertion allows the correct folding of the domain, suggesting that this domain binds dsRNA *in vivo* in the context of full-length protein.

The insertion in domain 2 is required for Stau–osk mRNA localization

To determine what role, if any, the extended loop in dsRBD2 plays in Stau function, we constructed a transgene (Stau^aloop^2) in which the normal domain 2 is replaced by the truncated dsRBD2aloop2 described above, and crossed this into a *stau*^D3^ mutant background. Although this transgene expresses high levels of a protein of the appropriate molecular weight (Figure 6B), it gives little or no rescue of the *stau* posterior phenotype; almost all osk mRNA and Stau protein fail to be transported to the posterior of the oocyte and remain trapped instead at the anterior margin (Figure 4A–E). However, a small amount of mRNA is occasionally seen at the posterior at stage 9. As a result of this defect in osk mRNA localization, none of the progeny of these flies hatch into larvae, and almost all develop less than one abdominal segment (Table I).

A trivial explanation for the inability of Stau^aloop^2 to

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Table I. Rescue of the *stau* null phenotype by *stau* transgenes
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Fig. 3. dsRBDs 2 and 5 do not bind to dsRNA in vitro. (A) A Coomassie-stained SDS–PAGE gel showing the expression of the Staufen dsRBDs fused to glutathione-S-transferase (GST). Lane 1, GST alone; lane 2, GST–dsRBD1; lane 3, GST–full-length dsRBD2; lane 4, GST–dsRBD3; lane 5, GST–dsRBD4; lane 6, GST–dsRBD5; lane 7, GST–dsRBD2 in which the large insertion has been replaced by the short loop 2 from dsRBD3; lane 8, GST–dsRBD3 containing five amino acid substitutions in residues that contact dsRNA (Ramos et al., 2000). (B) A Northwestern blot of the same samples as in (A) probed with [32P]dsRNA. The right hand side of this blot has been exposed approximately four times longer than the left to reveal the weak dsRNA-binding activity of dsRBD4. (C) A comparison of the RNA-binding faces of dsRBDs 1, 3, 4 and 5, showing the amino acids in domain 3 that are required for dsRNA binding (yellow boxes), and the identity and conservation of the amino acids in equivalent positions in the other domains (yellow). The structures of dsRBDs 1, 4 and 5 have been modelled by ‘threading’ them onto the known structure of dsRBD3 (Bycroft et al., 1995). Blue, basic residues; red, acidic; yellow, non-polar; orange, polar and uncharged. The amino acids are numbered from the first conserved residue of the domain (Ramos et al., 2000).

localize osk mRNA is that the removal of the extended loop in dsRBD2 disrupts the folding of the protein and prevents it from binding to the RNA. While it is not possible to test the binding of full-length Stau to osk mRNA in vitro, three lines of evidence suggest that this is not the case. First, dsRBD2Δloop2 binds to RNA in vitro, whereas the wild-type domain does not, indicating that this deletion facilitates the folding of the domain into the RNA-binding configuration. Secondly, StauΔloop2 co-localizes with osk mRNA to the anterior of the oocyte (Figure 4F). The localization of Stau to the oocyte requires its association with osk mRNA, since the protein remains in the nurse cells in an osk mRNA null mutant (M. Weston and D. St Johnston, unpublished results). The normal co-localization of the mutant protein to the anterior of the oocyte with osk mRNA therefore indicates that it still binds to the RNA.

The third argument to suggest that StauΔloop2 interacts with osk mRNA makes use of the oskBRE- transgene to uncouple osk mRNA translation from localization. In wild-type ovaries, the ectopic Osk protein produced from oskBRE- mRNA suppresses head development in about half of the embryos, and causes a duplication of abdominal segments at the anterior (bicaudal phenotype) in over a third (Table I). Even though the translation of this mRNA does not require its localization to the posterior, it is not efficiently translated in the absence of Stau protein: none of the embryos laid by stauid3;oskBRE- mothers develop the bicaudal phenotype, and almost all form only one abdominal segment at the posterior (Figure 4G). In contrast, StauΔloop2 activates translation of oskBRE- mRNA almost as effectively as wild-type Stau: 36% of the embryos laid by StauΔloop2 stauid3;oskBRE- females develop a bicaudal phenotype, and another third show a suppression of head development and partial or complete rescue of the abdomen (Figure 4H and I). Thus, while StauΔloop2 is unable to localize osk mRNA, it is able to activate its translation, providing strong evidence that the protein is bound to the mRNA. This mutant protein therefore retains some of the functions of wild-type Stau, but has lost the ability to mediate the transport of Stau–osk mRNA complexes from the anterior to the posterior of the oocyte.

Domain 5 is required to activate the translation of osk mRNA at the posterior pole

To determine which functions of Stau are mediated by dsRBD5, we constructed a transgene in which the C-terminus of the protein is deleted (StauΔdsRBD5). When introduced into flies lacking wild-type Stau, StauΔdsRBD5 completely rescues the posterior localization of osk mRNA, and co-localizes with the RNA to the posterior (Figure 5A–D). Thus, the mutant protein retains the ability both to
bind osk mRNA and interact with the factors required for transport to the posterior. However, this construct shows almost no rescue of the stauD3 abdominal phenotype, and neither osk mRNA nor StauΔdsRBD5 protein are localized at the posterior by the time the egg is laid (Table I; data not shown). These observations show that dsRBD5 is required for a function of Stau that occurs after osk mRNA localization, suggesting that it may play a role in the translation of Osk protein. In wild-type ovaries, localized osk mRNA is translated to produce a tight posterior crescent of Osk protein at stage 10a (Figure 5E). In contrast, no detectable Osk is produced at the posterior of StauΔdsRBD5 oocytes, even though osk mRNA is correctly localized at this stage (Figure 5F). This requirement of StauΔdsRBD5 for osk mRNA translation explains the failure of this transgene to rescue the development of the abdomen, and also accounts for the loss of osk mRNA and Stau from the posterior at later stages of oogenesis, since Osk protein has been shown to be necessary for the anchoring of Stau–osk mRNA complexes (Ephrussi et al., 1991; Kim-Ha et al., 1991).

To examine the role of dsRBD5 in the regulation of osk mRNA translation further, we crossed the StauΔdsRBD5 transgene into the stauD3;oskBRE- background. Like StauΔloop2, StauΔdsRBD5 activates the translation of de-repressed osk mRNA. The resulting embryos develop an average of 5.2 abdominal denticle belts, compared with 1.3 for stauD3;oskBRE- alone, and the majority show head defects caused by anterior Osk activity (Table II). This construct differs from StauΔloop2, however, in that it causes a lower frequency of bicaudal embryos, and this may be because osk mRNA is localized to the posterior rather than the anterior of the oocyte. Thus, StauΔdsRBD5 can activate the localization-independent translation of oskBRE- mRNA, but not the translation of wild-type osk mRNA at the posterior pole, suggesting that dsRBD5 is specifically required for the activation of translation once the mRNA has been localized.

bcd mRNA localization requires dsRBD2 and dsRBD5

Since Stau is also required for the anterior anchoring of bcd mRNA, we examined whether the StauΔdsRBD5 and StauΔloop2 transgenes could rescue the bcd mRNA localization defect of a stau null mutation. Although both mutant proteins are expressed at similar levels to endogenous Stau, neither anchors bcd mRNA at the anterior (Figure 6A and B). Surprisingly, both constructs almost completely rescue the stau head phenotype, even though they do not restore the wild-type localization of the bcd mRNA. Whereas 100% of the embryos laid by stauD3 homozygous females at 18°C lack all or part of the head skeleton, over two-thirds of the embryos laid by the transgenic stauD3 females have wild-type heads, and the rest have much milder head defects than in stauD3 alone (Table I). This suggests that Stau plays a second role in the regulation of bcd mRNA expression that is independent of its function in localization. In contrast to its role in anchoring, this activity does not require dsRBD5 or the insertion in dsRBD2.

Discussion

The only regions of Stau to have been maintained through evolution are the dsRBDs and a short region within the

Fig. 4. The insertion in dsRBD2 is required for the posterior localization of osk mRNA. (A and B) Wild-type stage 9 and 10A egg chambers showing the normal localization of osk mRNA to the posterior of the oocyte. (C and D) stauD3 T[StauΔloop2]/stauD3 egg chambers, in which all osk mRNA remains anchored at the anterior of the oocyte. (E) A stauD3 T[StauΔloop2]/stauD3 stage 9 egg chamber, showing a small amount of osk mRNA at the posterior. (F) StauΔloop2 protein (yellow) co-localizes with osk mRNA to the anterior of the oocyte. (G) A cuticle preparation of a typical embryo from a stauD3;oskBRE- female, with a normal head and only one abdominal segment. (H and I) Typical embryos from StauΔloop2 stauD3;oskBRE- females, showing the loss of head structures and rescue of the abdomen (H), and the stronger symmetric bicaudal phenotype (I).
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Fig. 5. dsRBD5 is required for osk mRNA translation. (A–D) Both StauΔdsRBD5 protein (A and B) and osk mRNA (C and D) localize normally to the posterior of stage 9 (A and C) and 10 oocytes (B and D). (E and F) Osk antibody stainings of wild-type (E) and stauD3 T[StauΔdsRBD5]/stauD3 oocytes (F).

Table II. StauΔloop2 and StauΔdsRBD5 activate the translation of osk BRE

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<th>Genotype</th>
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<th>% anterior defects with full or partial abdomen</th>
<th>% bicaudal</th>
<th>% stauD3-like</th>
<th>Average No. of abdominal denticle belts</th>
</tr>
</thead>
<tbody>
<tr>
<td>+:oskBRE−</td>
<td>11</td>
<td>53</td>
<td>36</td>
<td>0</td>
<td>6.5</td>
</tr>
<tr>
<td>stauD3;oskBRE−</td>
<td>1</td>
<td>5a</td>
<td>0</td>
<td>93</td>
<td>1.3</td>
</tr>
<tr>
<td>stauD3 T[StauΔloop2];oskBRE−</td>
<td>7</td>
<td>33a</td>
<td>36</td>
<td>24</td>
<td>5.0</td>
</tr>
<tr>
<td>stauD3 T[StauΔdsRBD5];oskBRE−</td>
<td>5</td>
<td>52a</td>
<td>9</td>
<td>34</td>
<td>5.2</td>
</tr>
</tbody>
</table>

*Embryos were only assigned to this class if their head defects were stronger than those seen in stauD3 alone, to distinguish between the phenotype caused by the stau defect in bcd mRNA localization, and that caused by the ectopic anterior expression of Oskar protein.

insertion in dsRBD2. Furthermore, the dsRBD-containing region from M.domestica retains all the essential functions of Stau during oogenesis. Thus, these conserved domains are likely to mediate all of the activities of Stau, such as binding to bcd and osk mRNAs, and its interactions with the factors that mediate mRNA transport and translational control. The alignment of the dsRBDs from all six species shows that natural selection is acting to maintain the unique characteristics of each. Consistent with this, only domains 1, 3 and 4 bind to dsRNA in vitro, while domains 2 and 5 are required for other activities of Stau.

The presence of a loop splitting dsRBD2 is the most pronounced conserved feature of all the Stau homologues. Replacement of this loop with the corresponding residues from dsRBD3 disrupts the posterior localization of almost all Stau and osk mRNA complexes, although a small amount is sometimes seen at the posterior of a few oocytes. A very similar variable localization of trace amounts of osk mRNA is also seen in all other mutants that specifically disrupt osk mRNA localization, such as mago nashi and Tropomyosin II, suggesting that this RNA may reach the posterior by a parallel translation-dependent pathway (Newmark and Boswell, 1994; Erdélyi et al., 1995). Wild-type osk mRNA is only translated once it is localized, and the resulting Osk protein anchors its own mRNA and Stau at the posterior pole. Thus, any RNA that diffuses
to the posterior should be trapped by localized translation and anchoring, and this could localize a tiny fraction of the mRNA in the absence of the normal transport pathway. Despite its inability to localize osk mRNA to the posterior, StauΔloop2 still associates with the RNA at the anterior of the oocyte, and activates the translation of oskBRE::RNA. These observations strongly suggest that StauΔloop2 interacts with osk mRNA, but that the removal of the insertion in dsRBD2 prevents the resulting complexes from associating with the transport machinery. This insertion probably disrupts the binding of the domain to dsRNA in vitro by changing the relative positions of the RNA-binding amino acids on either side. The domain binds dsRNA when the insertion is removed, however, and the domain contains several of the amino acids that contact RNA in other dsRBDs. Since these residues have been maintained by natural selection, it seems very likely that the domain interacts with RNA in vivo in the context of the full-length protein. Indeed, it is easy to envisage that the interaction of dsRBDs 1, 3 and 4 with RNA presents a very high local concentration of dsRNA to dsRBD2, which induces the domain to adopt the RNA-binding configuration.

Stau localization in both the oocyte and early embryo requires its association with the appropriate mRNA, suggesting that the protein undergoes a conformational change on binding RNA that allows it to associate with the factors that mediate RNA transport. Since the insertion in dsRBD2 is required for localization, it is attractive to propose that this conformational change occurs in dsRBD2. The two halves of dsRBD2 must come together for the domain to bind RNA, and this should loop out the insertion, which could then interact with the factors that transport Stau-RNA complexes. Thus, dsRBD2 could act as a conformational switch that senses the presence of bound RNA, and couples these complexes to the localization machinery. Alignment of the loops in dsRBD2 from different species reveals that they are highly divergent in both sequence and length, but there is a short block of amino acids that might represent a conserved motif with which these transport factors could interact (Figure 1B and C).

While the microtubule-dependent localization of osk mRNA requires the insertion in dsRBD2, but not dsRBD5, the converse is true for the actin-dependent localization of prospero mRNA in embryonic neuroblasts. In this case, localization is mediated by the binding of Miranda protein to dsRBD5 (Fuerstenberg et al., 1998; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998). Thus, distinct domains of Stau mediate microtubule- and actin-dependent mRNA localization, presumably by recruiting different trans-acting factors. Although Stau is the first example of an RNA-binding protein that can direct localization along both actin and microtubules, it is likely that other proteins will also have this capacity. Chicken ZBP-1 protein, which binds to part of the β-actin mRNA localization sequence, is the homologue of VERA/Vg1RBP, which binds to the localization element that directs the microtubule-dependent localization of Xenopus Vg1 mRNA (Ross et al., 1997; Deshler et al., 1998; Havin et al., 1998). Thus, this protein is implicated in both microtubule- and actin-based localization, albeit in different organisms, but it remains to be seen whether distinct domains of the protein are required for each process.

While the localization of osk and prospero mRNAs requires either dsRBD5 or the insertion in dsRBD2, both domains are necessary for the Stau-dependent anchoring of bcd mRNA at the anterior of the egg. Very little is known about the steps in bcd mRNA localization that occur at the end of oogenesis, because it has been impossible to visualize the distribution of the RNA once the vitelline membrane is deposited around the egg. However, these results raise the possibility that Stau needs to interact with both the microtubule and actin cytoskeletons to anchor bcd mRNA.

**Role of Stau in translational control**

It has previously been difficult to investigate the role of Stau in osk mRNA translation for two reasons. First, stau
null mutations disrupt the localization of osk mRNA, and it is not translated unless it is localized to the posterior pole (Markussen et al., 1995; Rongo et al., 1995). Second, it is difficult to distinguish between the effects of weak stau alleles on translation and anchoring, because Osk protein is required to anchor its own RNA, but the mRNA needs to be anchored at the posterior to be translated (Ephrussi et al., 1991; Kim-Ha et al., 1991). However, Stau<sup>ΔRBD5</sup> seems to have a specific defect in osk mRNA translation, as osk mRNA is localized normally to the posterior at stage 10 in these ovaries, but no detectable Osk protein is produced. Furthermore, oskBRE<sup>−</sup> RNA produces significant amounts of Osk activity in these ovaries, indicating that Stau<sup>ΔRBD5</sup> can function in the translation of derepressed osk mRNA. Taken together, these results strongly suggest that dsRBD5 is required to relieve Bruno repression once the mRNA has reached the posterior. This requirement cannot be absolute, however, since some Osk protein must be present early in oogenesis to anchor Stau–osk mRNA complexes.

Since dsRBD5 does not bind RNA, it presumably mediates its function in Osk translation through protein–protein interactions. Although Miranda binds to this domain, this interaction is unlikely to play any role during oogenesis, since miranda null germ line clones have no phenotype (Matsuzaki et al., 1998). Thus, dsRBD5 presumably interacts with other proteins to regulate osk translation. A very similar translation defect is observed in osk transgenes that lack binding sites for 68 and 50 kDa proteins in the 5′ UTR, whereas Stau is thought to associate with the localization signal in the 3′ UTR (Gunkel et al., 1998). Thus, derepression is likely to involve cooperation between proteins bound to both ends of the RNA.

In addition to its role in derepressing osk translation at the posterior, Stau is required for the efficient expression of derepressed oskBRE<sup>−</sup> RNA. Since neither the insert in dsRBD2 nor dsRBD5 is necessary for this activity, it presumably depends on the dsRBDs that bind RNA. It is possible that these dsRBDs also interact with other proteins, since only one face of the domain contacts RNA, and several amino acids on the other faces of these domains have been conserved during evolution. Alternatively, the binding of Stau may enhance osk mRNA translation indirectly, for example, by altering the folding of the RNA so that other factors can bind more efficiently.

Both Stau<sup>Δloop2</sup> and Stau<sup>ΔRBD5</sup> partially rescue the stau head phenotype, even though they do not restore the wild-type localization of bcd mRNA. Thus, more Bcd activity must be produced from the mislocalized mRNA in the presence of these mutant proteins than in stau<sup>D3</sup> alone, indicating that they provide a function of Stau that is independent of its role in anchoring. A comparison of the phenotypes produced by vasa exu and stau exu double mutants also indicates that Stau has a second function in the regulation of bcd mRNA. exu mutants block the localization of bcd mRNA early in oogenesis, and result in a uniform distribution of the RNA along the anterior–posterior axis of the embryo, while both vasa and stau mutants prevent the formation of the pole plasm, and therefore lack Nanos activity, which represses bcd mRNA translation (St Johnston et al., 1989; Wang and Lehmann, 1991; Wharton and Struhl, 1991; Wang et al., 1994). Despite the identical distributions of bcd RNA in these genotypes, vasa exu embryos develop anterior head structures everywhere, indicating that they contain high levels of Bcd activity, whereas stau exu form only thoracic structures (Schüpbach and Wieschaus, 1986). Thus, the removal of Stau reduces the level of Bcd expression, in the absence of any effect on mRNA localization. We can envisage two explanations for this localization-independent function of Stau. Stau binding could protect bcd RNA from degradation, and therefore increase the total amount of RNA. Alternatively, Stau could enhance the efficiency of bcd translation, in much the same way as it does for osk mRNA.

Since Stau has been conserved throughout animal evolution, it seems likely that the homologues will fulfill similar functions in mRNA localization and translational control in other organisms. In support of this view, recent evidence indicates that mammalian Stau mediates mRNA transport along microtubules in neurons (Köhmann et al., 1999). The mouse and human Stau genes share an extra region of homology not found in the insect homologues, which resembles the microtubule-binding domain of MAP1B, and this region of HsStau binds to microtubules in vitro (Marion et al., 1999; Wickham et al., 1999). It will therefore be interesting to see whether this domain or the insertion in dsRBD2 is required for the microtubule-dependent movement of Stau in neurons.

Materials and methods

**Cloning Stau homologues**

*Drosophila viridis* and *M.domestica* homologues were obtained by low stringency screens of genomic libraries (Thummel, 1993; Curtis et al., 1995). These sequences have been submitted to the DDBJ/EMBL/GenBank under accession Nos AF225924 and AF225925. The human homologue (DDBJ/EMBL/GenBank accession No. T06248) was identified in the EST database (Adams et al., 1993). The corresponding clone (HFBDQ83) was obtained from the American Type Culture Collection (ATCC) and sequenced. The mouse homologue was obtained by low stringency screening of a 7.5 d.p.c. mouse embryonic cDNA library (L. Lue, unpublished) with HFBDQ83. Cestau was identified in cosmid F55A4 by BLAST searching, and corresponds to the predicted gene F55A4.5. ESTs directly confirm 6/10 of the predicted splice junctions. All but one of the remaining splice sites were confirmed by direct examination of the sequencing traces.

**Drosophila mutants and transgenic lines**

The oskBRE<sup>−</sup> stock was a kind gift from Paul MacDonald. stau<sup>D3</sup> is a protein null (St Johnston et al., 1991).

Transgenes were expressed from a P-element transformation vector, pCaTUBmysSTOP, which drives maternal germline expression from the α tubulin promoter (Micklem et al., 1997). The Stau<sup>Δall</sup>, Stau<sup>ΔRBD5</sup> and Stau<sup>Δloop2</sup> constructs are identical to D288, Stau<sup>ΔRBD5</sup> and Stau<sup>ΔRBD2</sup> described previously (Schuldt et al., 1998). Stau<sup>Δall</sup> was generated in a similar manner, but lacks the first 281 aa of Stau, upstream of the Clal site at position 1140. Stau<sup>Δmd</sup> is identical to Stau<sup>Δall</sup> from the start codon to 6 aa before the start of dsRBD1 (up to and including the sequence TSSSGRG). This portion is fused to the dsRBDs of MdStau starting at an Xmal site 4 aa before the start of dsRBD1 (i.e. from REKTPMCCLV). A single Ala residue replaces the Gly of DmStau (or the Ser of MdStau) 5 aa before the start of dsRBD1. Further details of these constructs are available on request.

**Northwestern blots**

Northwestern blots were performed using the procedure described in St Johnston et al. (1992), using a blocking buffer of 2.5% (v/v) Tween-20, 1% (w/v) milk, 25 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM HEPES pH 8.0, 100 μM EDTA, 1 mM dithiothreitol (DTT), and a binding buffer of 2.5% Tween-20, 1% milk, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM HEPES pH 8.0, 100 μM EDTA and 1 mM DTT containing 500 000 c.p.m/ml of probe.
Antibody stainings and in situ hybridizations were performed as described in St Johnston et al. (1991). Antibodies were used at the following dilutions: rabbit α-Stau, 1/1000; rabbit α-Osk, 1/200.

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