The *Drosophila hairy* RNA localization signal modulates the kinetics of cytoplasmic mRNA transport

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In several *Drosophila* cell types, mRNA transport depends on microtubules, the molecular motor dynein and *trans*-acting factors including Egalitarian and Bicaudal-D. However, the molecular basis of transcript recognition by the localization machinery is poorly understood. Here, we characterize the features of *hairy* pair-rule RNA transcripts that mediate their apical localization, using *in vivo* injection of fluorescently labelled mRNAs into syncytial blastoderm embryos. We show that a 121-nucleotide element within the 3'-untranslated region is necessary and sufficient to mediate apical transport. The signal comprises two essential stem-loop structures, in which double-stranded stems are crucial for localization. Base-pair identities within the stems are not essential, but can contribute to the efficiency of localization, suggesting that specificity is mediated by higher-order structure. Using time-lapse microscopy, we measure the kinetics of localization and show that impaired localization of mutant signals is due to delayed formation of active motor complexes and, unexpectedly, to slower movement. These findings, and those from co-injecting wild-type and mutant RNAs, suggest that the efficiency of molecular motors is modulated by the character of their cargoes.

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Introduction

Asymmetric mRNA localization is evident in diverse cell types in many organisms, and is used to distribute protein products to appropriate regions of the cytoplasm (reviewed by Kloc *et al.*, 2002). Localization depends on *cis*-acting RNA elements (localization signals) that usually reside in untranslated regions (UTRs) of the transcripts, and which direct association with *trans*-acting protein factors. The structural basis of transcript recognition by the localization machinery is not well defined, although RNA secondary structure is known to be important in several cases (e.g. Macdonald, 1990; Serano and Cohen, 1995; Ainger *et al.*, 1997; Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999).

The early embryo of the fruitfly *Drosophila melanogaster* is particularly well suited to studying the mechanisms of asymmetric RNA localization. During early development, the embryo is a syncytium within which a peripheral

layer of several thousand nuclei share a single cytoplasm surrounding a central yolk mass. Several transcripts localize exclusively apically of the peripheral nuclei, via signals in their 3'UTRs. These include transcripts of the pair-rule segmentation genes (Davis and Ish-Horowicz, 1991), which are expressed in stripes and establish segmental organization in the embryo, and the *wingless* (*wg*) segment polarity gene (Simmonds *et al.*, 2001). Recent advances in characterizing components of the localization machinery have come from a rapid assay in which fluorescently labelled transcripts localize apically following injection into the cytoplasm of syncytial blastoderm embryos. Localization of injected transcripts is mediated by rapid microtubule-dependent transport (Lall *et al.*, 1999; Bullock and Ish-Horowicz, 2001; Wilkie and Davis, 2001). Transport is directed towards the minus ends of the microtubules, and uses the dynein/dynactin motor complex (Wilkie and Davis, 2001). The same machinery appears to transport several mRNAs into the nascent oocyte from the neighbouring, interconnected nurse cells (Swan *et al.*, 1999; Bullock and Ish-Horowicz, 2001). These maternal transcripts localize apically on injection into embryos, and RNA signals from pair-rule transcripts drive transport of maternally expressed transcripts into the oocyte (Bullock and Ish-Horowicz, 2001).

In embryogenesis and oogenesis, the transport machinery includes the Egalitarian (Egl) and Bicaudal-D (BicD) proteins (Bullock and Ish-Horowicz, 2001), which are complexed together *in vivo*, and are enriched at the minus ends of microtubules (Mach and Lehmann, 1997; Bullock and Ish-Horowicz, 2001; Oh and Steward, 2001). Egl and BicD are specifically recruited *in vivo* to localizing transcripts prior to and during transport, and their activities are required for localization of injected and endogenous mRNAs (Bullock and Ish-Horowicz, 2001). Where tested, recruitment of Egl and BicD to these transcripts is mediated by RNA localization signals and it is possible that Egl and/or BicD contact the transcripts directly. However, neither protein possesses a known RNA-binding motif (Suter *et al.*, 1989; Wharton and Struhl, 1989; Mach and Lehmann, 1997) or has yet been shown to bind RNA directly. A mammalian BicD homologue (BICD2) has roles in membranous organelle trafficking and interacts directly with the dynamitin subunit of dynactin (Hoogenraad *et al.*, 2001), suggesting that this protein has a conserved role in linking cargoes to dynein.

The structural basis of the RNA signals that mediate recognition by the localization machinery is unclear. The *K10* and *bicoid* (*bcd*) transcripts rely on secondary structure, in particular double-stranded stems, for localization during oogenesis and in blastoderm embryos, and for *in vivo* recruitment of Egl and BicD (Serano and Cohen, 1995; Macdonald and Kerr, 1997; Bullock and Ish-Horowicz, 2001). The primary sequences of the *K10* and

bcd signals are not obviously related to one another, or to any of the pair-rule 3'UTRs. It is therefore not clear whether different mRNAs are bound by unique RNA-binding proteins, or whether they share a cryptic motif that is recognized by a common factor.

To investigate the mRNA recognition events involved in localization in more detail, we have mapped and characterized the *cis*-acting mRNA element that mediates apical localization of the *hairy* (*h*) pair-rule transcript and recruitment of Egl and BicD in the blastoderm embryo. By extensive mutational analysis and evolutionary sequence comparison, we demonstrate that this signal consists of two partially redundant stem-loop structures. Our results implicate higher-order RNA structure in signal recognition by the motor machinery. We also use time-lapse microscopy to describe in detail the transport characteristics of wild-type and weakly localizing mutant transcripts. The latter take longer to form active transport complexes and are transported more slowly. These findings provide evidence that the activity of motors is modulated by the character of their cargoes.

Results

Mapping the *h* localization element

Previous experiments using transgenic flies have shown that the *h* 3'UTR includes sequences required to localize *h*

transcripts apically in blastoderm embryos (Davis and Ish-Horowicz, 1991). We confirmed this result by examining the localization of fluorescently labelled RNA 5–8 min after injection into the basal cytoplasm of blastoderm embryos. In this system, injected *D.melanogaster h* transcripts accumulate exclusively in the apical cytoplasm in >90% of embryos (Figure 1A; Bullock and Ish-Horowicz, 2001), whereas transcripts lacking the 3'UTR (*hΔ3'*) never display apical enrichment (Figure 1A). Injection of fluorescent RNA derived from further deletion constructs, *h-Sall5'*, *h-Sall3'* and *h-NsiI3'*, indicates that the localization signal resides in the 5'-half of the 3'UTR, between positions 1171 and 1406 (Figure 1B).

Analysing a series of internal deletions in this region defines a region between positions 1281 and 1406 that is required for apical transport of *h* (Figure 1C). *h* transcript localization is unaffected by deletions outside this region (e.g. *hΔA*, *hΔB*, *hΔC*), whereas most transcripts with deletions that encroach on this region localize very poorly (*hΔF*, *hΔG*, *hΔH*; apical transcript accumulation is weak, and only detectable in ~10–20% of embryos) or not at all (*hΔD*, *hΔE*, *hΔE2*). This region is also required *in vivo* for localization of endogenous transcripts; a full-length *h* transcript with the *hΔD* deletion fails to localize apically in blastoderm embryos (S.L.Bullock and D.Ish-Horowicz, unpublished data). It is also sufficient to mediate apical localization of injected *string* (*stg*) reporter transcripts that are otherwise unlocalized (Figure 1A and D). Together, these results delimit the *h* localization element (HLE), which is both necessary and sufficient for recognition by the localization machinery.

Nevertheless, its activity is context dependent: *stg-HLE* transcripts localize less efficiently than *h-Sall5'* transcripts, as though the region 5' to the HLE includes a weak supplementary signal that enhances its activity. The HLE is also fully efficient in the context of the entire 3'UTR (*h-NsiI3'*), indicating that supporting sequences are redundant, lying both 5' and 3', but lacking autonomous activity (e.g. *hΔD*). However, we cannot exclude the possibility that the *stg* reporter includes inhibitory sequences.

Levels of Egl and BicD proteins are elevated at sites of injected, localized transcripts, including *h* (Figure 2; Bullock and Ish-Horowicz, 2001). This localization reflects protein recruitment prior to transport because it is seen before most RNA translocation has occurred

Fig. 1. Mapping the *h* localization element. (A) Confocal images of representative blastoderm embryos injected with fluorescently labelled transcripts, illustrating examples of wild-type localization (*h*; ++), no localization (*hΔ3'*; -) or weak localization (*stg-HLE*; +). Arrowhead indicates the approximate site of injection. Apical is at the top of this and other figures. (B) Truncated *h* transcripts map the HLE to between nt 1171 and 1406 (+/- represents very weak apical transcript enrichment in 10–20% of injected embryos). (C) Internal deletions across this region map the HLE to nt 1281–1406. (D) The HLE is sufficient to mediate localization of *stg* reporter transcripts. Scale bar: 50 μm.

Fig. 2. Recruitment of Egl and BicD to the HLE. Immunostaining to reveal distribution of Egl (green) and BicD (blue) following injection of *h*, *hΔD* or *hΔG* transcripts (red). Weak apical recruitment of Egl and BicD to *hΔG* transcripts (which have detectable apical enrichment in 10–20% of embryos) is observed, indicating that a small proportion of the injected transcripts accumulated apically by active transport. Scale bar: 50 μm.

(1–2 min), and even when the microtubules are disrupted (Bullock and Ish-Horowicz, 2001). Recruitment of Egl and BicD is abolished by inactivating mutations of the HLE (e.g. *hΔD*; Table I), and significantly reduced for weakly localizing mRNAs (*hΔG*; Figure 2). Thus, protein

recruitment to *h* is mediated by the HLE and is intimately associated with transport.

Evolutionary conservation of the HLE

To investigate the structural basis of the HLE in more detail, we examined its probable secondary structure using the algorithm mfold (<http://bioinfo.math.rpi.edu/~mfold/rna>; Mathews *et al.*, 1999; Zuker *et al.*, 1999), which predicts RNA secondary structure based on free-energy minimization. mfold calculated several putative, unrelated structures for the HLE (data not shown), none of which matched structures derived from analysing other localization signals.

As an alternative way of finding functionally important structural features of the HLE, we studied its evolutionarily conservation. We cloned the *h* 3'UTRs from *Drosophila willistoni*, *Drosophila nebulosa*, *Drosophila azteca* and *Drosophila virilis*, which diverged from *D.melanogaster* between 40 and 60 million years ago (Beverley and Wilson, 1984). Fluorescent transcripts

Table I. Recruitment of Egl and BicD by HLE variants

Transcript	Localization	Recruitment of Egl and BicD
<i>h</i>	++	+
<i>hΔD</i>	–	–
<i>hΔG</i>	+/-	+ ^a
<i>d22</i> (class I)	–	–
<i>e33</i> (class II)	+	+ ^a
<i>g15</i> (class II)	+	+ ^a

Recruitment was assayed 5–8 min following RNA injection.

^aWeak recruitment, predominantly to RNA that is concentrated apically. See Figure 1 for classification of localization efficiency.

Fig. 3. Phylogenetic analysis of the HLE. (A) Localization of *h* 3'UTR sequences from divergent *Drosophila* species upon injection into *D.melanogaster* embryos. Scale bar: 50 μ m. (B) Alignment of the RNA sequence of the *D.melanogaster* HLE with the corresponding region of the *h* 3'UTR from other *Drosophila* species. Identical and conserved nucleotides are highlighted in black and grey, respectively. The internal deletions (*hΔD-H*) of the *D.melanogaster* HLE are indicated. Dashed lines and thick underscores highlight the sequences forming SL1 and SL2a and the regions mutated in *hr21* and *hr22* (see Figure 4), respectively. (C) Predicted RNA secondary structures of highly conserved nt 1286–1331 in *D.melanogaster h* and corresponding sequences in other *Drosophila* species. Foldings were produced using ConStruct (Lück *et al.*, 1999) using Tinoco settings at 25°C. Nucleotides that differ from *D.melanogaster h* are highlighted in black. Base-pairing may exist between nucleotides in the illustrated single-stranded regions, although these are not predicted to be evolutionarily conserved.

Fig. 4. Characterization of SL1 and SL2a. Site-directed mutagenesis of (A) SL1 and (B) SL2, and representative examples of embryos injected with mutant transcripts as indicated. Mutations are boxed according to the degree of localization they promote. Black, no localization; dark grey, very weak localization in only ~10% of embryos; light grey, weak localization in all embryos; white, efficient localization. Mutations were tested in the context of the full-length *h* transcript. (C) Activity of multimers of SL1 (*h2xSL1*) or SL2a (*h2xSL2a*, *h4xSL2a* and *h6xSL2a*) within the context of the *h* 3'UTR in embryos fixed 5–8 min after injection. See Supplementary data for the full sequences of the regions used to replace the HLE in these mutants. Scale bar: 50 μ m.

derived from all the 3'UTRs localize efficiently on injection into *D.melanogaster* embryos (Figure 3A), indicating that they all retain the basis for apical localization. Also, *D.melanogaster h* transcripts localize efficiently upon injection into *D.virilis* embryos (data not shown), the most divergent species studied. These results indicate that the localization signal and machinery are conserved within *Drosophila* species.

We identified a putative domain of secondary structure present in all the species, using the ConStruct programme (<http://www.biophys.uni-duesseldorf.de/local/ConStruct/Construct.html>; Lück *et al.*, 1999), which calculates minimal energy foldings common to a group of related primary RNA sequences. All HLEs include a predicted 5' stem-loop motif, formed by conserved primary sequences [Figure 3B; nucleotides (nt) 1286–1331 in *D.melanogaster*], which we designate stem-loop 1 (SL1; Figure 3C).

SL1 is required for localization

The above deletion analysis of the *D.melanogaster* HLE is consistent with a role for SL1 in *h* localization. This structure is disrupted by *hAD*, *hAE* and *hAE2*, all of which

abolish apical localization, but not by *hAE3*, which retains activity (Figure 3B). We tested directly whether SL1 is required for localization *in vivo* by examining the behaviour of transcripts in which the predicted base-paired stem is disrupted. A series of 3 nt base transversions within the predicted stem (*hr11*, *hr12*, *hr13* and *hr14*) completely abolish localization of full-length *h* transcripts (Figure 4A). These results do not reflect primary sequence requirements for these bases because compensatory mutations (*hr11+12* and *hr13+14*), which are predicted to reform a base-paired stem with different primary sequences, restore localization activity in >90% of injected embryos (Figure 4A). These results indicate that signal specificity depends, at least in part, on the secondary structure of SL1. Nevertheless, the compensatory mutations do not restore full localization activity. Only a proportion of *hr11+12* and *hr13+14* transcripts localize within 5–8 min, indicating that primary sequence composition of SL1 plays a role in efficient recognition by the localization machinery. Requirements for other regions of SL1 were tested by random mutagenesis (see below).

Characterization of stem-loop 2

Analysis of the HLE deletion mutations indicates that the signal is bipartite: *hAE3* transcripts, which lack bases 1334–1351, localize normally, yet deletions of the region between 1356 and 1406 (*hAF*, *hAG* and *hAG2*) greatly impair transcript localization (Figure 1C). ConStruct did not reveal any evolutionarily conserved secondary structures downstream of SL1, but mfold predicted a thermodynamically favourable stem-loop structure between positions 1370 and 1411 in *D.melanogaster h*, which we designate stem-loop 2 (SL2; Figure 4B).

Mutational analysis supports the functional importance of the distal stem in SL2 (Figure 4B); disrupting predicted base pairing in this region (*hr21* or *hr22*) severely inhibits localization, leading to very weak localization similar to that exhibited by *hAF*, *hAG* and *hAG2* transcripts. The compensatory mutation *hr21+22* localizes much more efficiently, with most of the RNA localizing in >90% embryos, although transport is somewhat compromised because a significant proportion of transcripts is still basal after 5–8 min.

Analysis of additional mutations show that localization activity does not depend on the putative proximal stem or the apparently unpaired bases in SL2 (in bulges and the terminal loop) (summarized in Figure 4B). Together, these data indicate that the minimal region required for localization is no greater than 121 nt (between nt 1281 and 1402) and that SL2's activity lies within the double-stranded region of the distal portion, which we refer to as SL2a. Indeed, the sequences disrupted in *hr21* and *hr22* are more highly conserved than the rest of the SL2 sequences (Figure 3B).

The above data demonstrate that the HLE contains two important stem-loop structures: SL1, which has very weak autonomous activity (e.g. *hAG*), and SL2a, which is required for efficient localization but is inactive in isolation (e.g. *hAD*). However, the *hAF* deletion, which does not disrupt either structure directly, severely inhibits localization (Figure 1C). This is not because the stem-loops must be precisely spaced, as other deletions within the spacer (e.g. *hAE3*) localize normally. Either *hAF*

Fig. 5. Fine structural requirements for SL1 and SL2a. (A) Summary of class III mutations in SL1 (nt 1286–1331). Bases mutated by substitutions or deletions are highlighted in black. Asterisks represent the number of mutations at each position. Mutations that are likely to preserve base pairing that is similar to the wild-type structure (Supplementary figure 2) are boxed. (B) Structure of SL1 in *hSL1min*, which supports weak localization. See Supplementary data for the full sequence of the region used to replace the HLE for this mutant. (C) Single base substitutions in SL1 that disrupt localization. $1300^{d22}C \rightarrow G$ does not support localization; $1328^{e33}A \rightarrow U$ and $1316^{g15}A \rightarrow U$ support only weak localization. (D) Summary of class III mutations in SL2a (nt 1380–1402). Mutations are annotated as in (A). In addition, class III mutant *g7* contains an insertion of a G after position 1399 (data not shown; Supplementary figure 1). (E) Representative examples of embryos injected with transcripts as indicated. $1328^{e33}A \rightarrow U$ localization is indistinguishable from that of $1316^{g15}A \rightarrow U$ (not shown). Scale bar: 50 μ m.

causes misfolding of the signal, or there are additional sequences in this region that are recognized by the localization machinery.

Multimerization enhances the activity of SL1 but not of SL2a

SL1 and SL2a could co-operate by binding similar or different factors. In the former case, multimerizing either element would increase the number of protein–RNA contacts and might boost its activity. To test this hypothesis, we generated transcripts including multimers of either SL1 or SL2a in the context of the remainder of the *h* 3'UTR. A dimer of SL1 (*h2xSL1*) leads to localization that is indistinguishable to that of wild-type *h* transcripts (Figure 4C; and see below). Thus, SL1 includes all features required for recognition by the transport machinery. In contrast, di-, tetra- or hexamers of SL2a appear to be completely inactive (Figure 4C), arguing that this element plays a secondary role to SL1.

Random mutagenesis of the HLE

To characterize the sequence determinants of the HLE in more detail, we performed extensive random mutagenesis of the *D.melanogaster* signal. Mutant HLEs were derived from oligonucleotides synthesized with a 5% rate of base transversions (i.e. $A \rightarrow T$, $T \rightarrow A$, $C \rightarrow G$, $G \rightarrow C$), such that each HLE included an average of six point mutations. The experiment also generated a number of small deletions and insertions in the HLE.

Eighty-eight different mutant HLEs were assayed for localization in the context of the *h* 3'UTR, 5–8 min after injection. In most cases, three mutants were analysed simultaneously, after labelling with different fluorochromes. We classified the mutant HLEs into three groups according to their degrees of apical localization (Supplementary figure 1, available at *The EMBO Journal* Online): class I mutants (53 out of 88), which appear inactive; class II mutants (14 out of 88), in which apical accumulation is substantially weakened; and class III mutants (21 out of 88), which appear to localize with similar efficiency to the wild type. Localization efficiency was classed similarly whether transcripts were injected individually or in pools. Efficiency of transcript localization by mutant transcripts parallels the amount of Egl and BicD that accumulates apically (Table I).

Although the mutagenesis was not saturating, analysis of class III mutants reveals numerous sites within the HLE that are not required for efficient apical localization (Supplementary figure 1). For example, various mutants together with the $h\Delta E3$ deletion mutant imply that sequences between 1334 and 1356 are dispensable for transport.

SL1

Fifteen of the class III mutants include a total of 31 mutations in SL1 (Supplementary figure 1; Figure 5A), most (18) of which occur in regions predicted not to have evolutionarily conserved base pairing. Indeed deleting a large portion of the terminal loop does not discernibly affect localization (*e21*; Supplementary figures 1 and 2). To determine whether the unpaired bases in SL1 are important for contacting the localization machinery, we introduced base substitutions into the loop and deleted all other predicted single-stranded regions (Figure 5B). This transcript (*hSL1min*) localizes apically, albeit less efficiently than wild-type *h* (Figure 5E). Thus, predicted single-stranded regions are not essential for recognition, but they contribute either directly to recognition or indirectly to presentation of the double-stranded regions. In any case, the double-stranded regions of SL1 are major determinants of SL1 activity.

One class I mutant (*d22*), which fails to localize, and two class II mutants (*e33* and *g15*), which localize weakly, have single base substitutions in the stem of SL1, all other mutations being at sites that class III mutants have shown to be dispensable (data not shown). These bases in SL1 are indeed important for localization because introducing the changes into the *h* 3'UTR as individual point mutations ($1300^{d22}C \rightarrow G$, $1328^{e33}A \rightarrow U$ and $1316^{g15}A \rightarrow U$; Figure 5C) leads to localization indistinguishable from that of the original random mutants. However, some other changes in the stem do not disrupt localization. Five such mutations should still permit similar base pairing to the wild-type

Table II. Effects on particle motility of mutations in the HLE

Transcript	No. of embryos	No. of particles	No. of positions tracked	Speed ($\mu\text{m/s}$) (mean $\pm$ SEM)	Maximum speed ($\mu\text{m/s}$)	Speed of directed motion ($\mu\text{m/s}$) (mean $\pm$ SEM)	Frequency of directed motion (%) (mean $\pm$ SEM)
<i>wt</i>	11	188	5176	0.58 ± 0.02	2.13	0.76 ± 0.02	65 ± 2
<i>h2xSL1</i>	10	170	3626	0.64 ± 0.02	1.91	0.82 ± 0.02	74 ± 4
<i>I328^{e33}A</i> → <i>U</i>	7	189	9758	$0.43 \pm 0.02^{**}$	1.48	$0.67 \pm 0.02^{**}$	$48 \pm 4^*$
<i>wt</i> + <i>I328^{e33}A</i> → <i>U</i>	8	178	8143	$0.41 \pm 0.02^{**}$	1.38	$0.62 \pm 0.01^{**}$	$52 \pm 2^*$
<i>I316^{g15}A</i> → <i>U</i>	6	65	2360	$0.44 \pm 0.03^*$	1.05	$0.62 \pm 0.02^{**}$	56 ± 5
<i>hr11+12</i>	5	107	4372	$0.40 \pm 0.02^{**}$	1.15	$0.60 \pm 0.01^{**}$	52 ± 5

* $P < 0.05$; ** $P < 0.01$ [compared with wild-type *h* 3'UTR (*wt*)]. *h2xSL1*, *I328^{e33}A*→*U* and *I316^{g15}A*→*U* are in the context of the *h* 3'UTR, and *hr11+12* is in the context of the full-length *h* transcript (the wild-type full-length *h* transcripts behave similarly to the *h* 3'UTR; data not shown).

SL1 (Supplementary figure 2; Figure 5A), and eight are thermodynamically predicted to disrupt base pairs without destroying the overall secondary structure (Supplementary figure 1; Figure 5A; data not shown). These latter mutations define bases that are likely to be less important for recognition by the localization machinery.

SL2a

Thirteen out of 21 class III transcripts have mutations in SL2a (Supplementary figure 1). Many of the mutations are found in predicted single-stranded positions (nine out of 20; Figure 5D), and four of the mutations in the double-stranded stem reflect two sets of compensatory changes that restore secondary structure (*d19* and *e36*; Supplementary figure 2). However, seven of the other mutations reside in the region that larger-scale mutations (*hr21* and *hr22*) have shown to be double-stranded *in vivo* (Figure 4B). Thus, like SL1, SL2a can tolerate minor changes in nucleotide identities within its double-stranded regions.

Analysing the kinetics of *h* transcript localization *in vivo*

Transcripts containing mutant HLEs might localize poorly for several reasons: because they are defective in assembling a transport complex, in binding their microtubule substrate, in sustaining transport (i.e. processivity) or in apical anchorage. Studies of mRNA localization in fixed embryos cannot discriminate between these alternatives. We therefore used time-lapse microscopy to monitor transport of injected transcripts in living embryos. Using these methods, Wilkie and Davis (2001) have reported that injected fluorescent *ftz*, *runt* and *wg* transcripts rapidly assemble into particles that are transported along microtubules in a basal to apical direction (i.e. towards microtubule minus ends) by the cytoplasmic dynein motor.

Wild-type *h* transcripts

We also observed apical transport of injected wild-type *h* 3'UTR transcripts as particles. These were evident as soon as we began filming (after ~1 min), although appearance of particles was not always accompanied by directed transport. Localization was largely complete within ~6–7 min (Supplementary movie 1). The mean and maximum speeds of *h* 3'UTR particle transport are 0.58 ± 0.02 and $2.13 \mu\text{m/s}$, respectively (Table II). Particles cease large-scale movement once they reach the apical cytoplasm

(Supplementary movie 1), presumably because of either anchorage or continual active transport. Interestingly, mRNAs lacking a functional localization signal (e.g. *h-SalI3'*) can also form particles, but these are largely static or oscillatory and do not accumulate apically (Figure 6A; data not shown). Thus, particles do not necessarily represent active transport complexes.

Using our imaging techniques we have noted that most *h* particles move intermittently, with interspersed periods of 'directed' motion, in which transcripts undergo persistent apical motion and 'non-directed' motion, in which particles appear static or oscillatory (e.g. Supplementary movie 2; Figure 6A). Transport appears to be predominantly unidirectional, because persistent basal (microtubule plus end)-directed motion is detected only very rarely. However, our ability to visualize these, and other, events could be restricted by the limited spatial and temporal resolution of our microscopy in imaging weak fluorescent signals deep within the embryo.

By tracking individual particles during transport, we can distinguish net periods of directed and non-directed motion (Materials and methods). Approximately 65% of the time, *h* particles show directed motion with mean speed of $0.76 \pm 0.02 \mu\text{m/s}$ (Table II). The durations of directed and non-directed events vary greatly between particles (Figure 6A; data not shown), indicating that they are not terminated after a set time. Similar bimodal behaviour and speed of transport is also seen for injected *ftz* transcripts (unpublished data). The kinetics of localization of injected *h2xSL1* transcripts are similar to those of wild-type transcripts, with respect to both the timing at which transport is initiated and the speed of transport (Table II). Indeed, *h2xSL1* may be marginally more efficient than *h* transcripts in these processes. Like *h* transcripts, *h2xSL1* transcripts show very little motility once they reach their apical destination. Thus, two copies of SL1 per transcript function at least as well as the wild-type HLE.

Mutant transcripts

To investigate the localization of weakly localizing transcripts, we filmed embryos injected with transcripts containing a compensatory transversion (*hr11+12*) or individual point mutations (*I328^{e33}A*→*U* and *I316^{g15}A*→*U*) in SL1. These transcripts are also transported as particles in a predominantly apical direction,

although in each case localization is largely complete only after ~12–15 min (Supplementary movie 3).

Appearance of transcript particles is not obviously delayed; like wild-type particles, these were evident when we began filming (data not shown). In contrast, there is a striking lag in the onset of directional motility of the mutant RNA particles (Figure 6B). Thus, the mutant particles are severely impaired in their ability to form an active motor complex.

A further cause of delayed localization is that mutant cargoes are transported ~25–30% more slowly than wild-type transcripts ($P < 0.05$; Table II). The maximum speed of particle transport is also much reduced compared with wild type. Decreased transport efficiency is due partly to significantly reduced velocity (~10–20% less) during periods of directed apical transport ($P < 0.01$). $1328^{e33}A \rightarrow U$ particles also spend, on average, significantly less time undergoing directed motion than wild-type particles (48% compared with 65%; $P < 0.02$; e.g. Figure 6A; Supplementary movie 3). $1316^{g15}A \rightarrow U$ and

$hr11+12$ particles also undergo periods of directed movement less frequently, although this is of marginal statistical significance. Owing to our limited temporal resolution, it is possible that decreased processivity of the weak localizing RNAs contributes to their apparent decrease in velocity during directed motion. Despite their impaired transport kinetics, mutant particles appear to be retained normally at their apical destination (Supplementary movie 3); this was confirmed by particle tracking (data not shown). Therefore these transcripts are either anchored normally, or residual transport is sufficient to prevent basal diffusion.

Weakly localizing RNAs can be co-transported with, but not rescued by, wild-type transcripts

To test whether wild-type and weakly localizing RNAs would be transported in the same or separate particles, we co-injected equimolar amounts of h 3'UTR and $1328^{e33}A \rightarrow U$ transcripts labelled with different fluorochromes. Time-lapse imaging (Figure 6C) and analysis of fixed material (data not shown) demonstrated that these transcripts can be transported apically in the same particles. All of the particles that are large enough to be tracked in movies contain similar proportions of wild-type and mutant transcripts, consistent with these containing a large number of RNA molecules. Unexpectedly, such particles display transport kinetics indistinguishable from those containing $1328^{e33}A \rightarrow U$ alone (Figure 6B; Table II). Particles also localize similarly following injection of a 3-fold excess of wild-type h RNA over $1328^{e33}A \rightarrow U$ (data not shown), suggesting that inclusion of mutant RNAs can 'poison' transport of predominantly wild-type transcript particles.

Despite this behaviour of mixed-transcript particles, wild-type h localization is clearly more efficient than that of $1328^{e33}A \rightarrow U$ transcripts when a 1:1 ratio of the two transcripts is co-injected (Figure 6C and D). Analysis of fixed embryos 5–8 min after injection shows that the

Fig. 6. Real-time analysis of mRNA transport. (A) RNAs are transported intermittently. Tracks of apical–basal motion versus time of representative RNA particles showing predominantly directed (crosses; h 3'UTR) or non-directed motion (triangles; $1328^{e33}A \rightarrow U$), respectively. A h -*SallI*3' particle that lacks a localization signal is also plotted (circles) to indicate the extent of movement of non-localizing particles. Note that movement in the lateral (anterior–posterior) axis is not shown in these plots. Arrows and arrowheads indicate examples of prolonged periods of apical directed and non-directed motion, respectively. (B) Onset of transport of weakly localizing mRNAs is delayed. The y-axis shows the percentage of tracked particles that start directed motion within a specific time window. The data begin 60 s after injection (when imaging began), and is grouped into subsequent bins of 2 min. Imaging was stopped either when no particles were visible basally for >60 s or after 15 min, whichever came first. Total number of particles: wild-type h 3'UTR (wt), 188; $h2xSL1$, 170; $1328^{e33}A \rightarrow U$, 184; $wt + 1328^{e33}A \rightarrow U$, 177; $1316^{g15}A \rightarrow U$, 61; $hr11+12$, 107. The total RNA concentration was 1 μg/μl for each experiment (500 ng/μl each for $wt + 1328^{e33}A \rightarrow U$). (C) Co-injected h 3'UTR (green; wt) and $1328^{e33}A \rightarrow U$ 3'UTR (red; wt) and co-injected $1328^{e33}A \rightarrow U$ 3'UTR (green) transcripts 2.5 min after injection. Total apical accumulation of the wild-type transcript is more efficient than that of the mutant. This can also be seen in (C). Scale bar: 10 μm in (C); 60 μm in (D).

overall apical accumulation of wild-type RNA is only partially inhibited in these experiments (data not shown). This suggests that a significant proportion of wild-type RNA becomes enriched apically by efficient transport in small cargoes that we are unable to detect (see Discussion).

Discussion

The *h* transcript localization signal depends on secondary RNA structure

The blastoderm injection assay offers a much more rapid test of localization activity than previous assays using transgenic animals and *in situ* hybridization to examine steady-state RNA distributions. The injection assay is also more sensitive to small changes in localization efficiencies, especially when combined with the use of time-lapse microscopy in living embryos. These advantages have allowed us to characterize the structural features of the *Drosophila h* mRNA localization signal in detail, and to study the transport kinetics of wild-type and modified RNA cargoes.

Our results show that efficient recognition of the *h* transcript depends on two stem-loops, SL1 and SL2a; each is necessary for robust localization, but neither is sufficient alone. The mutagenesis data demonstrate that evolutionarily conserved double-stranded stems of SL1 and SL2a are indispensable for proper transcript transport. However, secondary structure of the stems is not the sole determinant of signal activity because transversions that alter base-pair identities lead to inefficient localization (e.g. *hr11+12*, *hr13+14* and *hr21+22*). In addition, although many predicted single-stranded regions are inessential for signal activity, a mutant in which all of these bases are removed or altered (*hSL1min*) localizes only weakly. Thus, higher-order RNA structure is also likely to be important for specific recognition by the localization machinery.

Specificity could reside in the tertiary conformation of the RNA, as has been demonstrated for various well-characterized RNA-protein interactions (reviewed in Hermann and Patel, 1999). Such three-dimensional structures would be difficult to infer solely from our mutagenesis data, especially because of the large assortment of potential non-canonical interactions between bases (Leontis and Westhof, 2001).

Intermolecular RNA interactions could also be involved in *h* mRNA recognition. Transcript oligomerization appears to be important for localization of *bcd* transcripts to the anterior of the late oocyte/early embryo (Ferrandon *et al.*, 1997), although the signals and *trans*-acting factors driving this transport process seem to be distinct from those acting in Egl/BicD-mediated early export into the oocyte (Ferrandon *et al.*, 1997; Macdonald and Kerr, 1997). Our studies of mutant RNA transcripts have not yet revealed evidence that oligomerization is necessary for transport. We inject mixtures of up to three different transcripts, and have never observed rescue of a non-localizing mutant RNA signal by a co-injected localizing transcript. Nonetheless, our time-lapse studies show transport of injected *h* RNA in particles that contain numerous transcript molecules, although our imaging is not sensitive enough to detect cargoes of individual molecules. Indeed, particle formation is not sufficient to

direct formation of an active transport complex: non-localizing mRNAs such as *h-SalI3'* are also found in particles.

Different cargoes share common modes of transcript recognition

Although *K10* and *bcd* localization signals share no obvious primary sequence similarities with the HLE, they share structural features, suggesting that they are recognized similarly. The *K10* localization signal (*K10TLS*) is only 44 nt long and, unlike the HLE, comprises only a single stem-loop region (Serano and Cohen, 1995); nonetheless, it recruits Egl and BicD (Bullock and Ish-Horowicz, 2001). *bcd* transcripts also harbour a stem-loop (the 57-nt stem-loop V) that is required for early transport from nurse cells into the oocyte (Macdonald and Kerr, 1997), and for apical localization of injected *bcd* transcripts in the embryo and their association with Egl and BicD (Bullock and Ish-Horowicz, 2001). Like *h* SL1 and SL2a, the activities of both the *bcd* and *K10* stem-loops rely heavily on double-stranded stems in which exact base-pair identities contribute to, but do not determine, efficient localization; base-pair transversions in all the stems can compromise the efficiency of localization (Serano and Cohen, 1995; Macdonald and Kerr, 1998; Bullock and Ish-Horowicz, 2001). In common with the *h* SL1, the *bcd* stem-loop V is not sufficient for localization, but is fully active when dimerized (Macdonald *et al.*, 1993).

The apparent complexity and redundancy of the HLE supports a model for signal recognition in which multiple protein-RNA contacts are needed for the formation of a specific, stable complex, as has been discussed by Macdonald and Kerr (1998) for the *bcd* localization signal. In the HLE, weak binding sites for the machinery may be distributed in SL1 and SL2a. Thus, transcripts with two *h* SL1 domains are at least as active as those with a wild-type HLE. SL2a may provide quantitatively weaker binding signals as it is unable to support any localization either alone or when multimerized. One possibility is that SL1 alone establishes low affinity interactions with the localization machinery, and binds with high affinity together with SL2. The same mode of recognition could also apply for *K10*, if, unlike *h* and *bcd*, the requisite sites are located within a single stem-loop.

Despite the overall similarities of the structural requirements for localization of *bcd*, *K10* and *h*, we are unable to identify significant shared base-pair identities within essential regions of the signals. We cannot exclude the possibility that different transcripts are recognized by distinct RNA-binding factor(s) and recruited to shared components of the machinery. However, the same localization signals are active in a variety of cell types (Karlin-Mcginness *et al.*, 1996; Bullock and Ish-Horowicz, 2001; J.R.Hughes, S.L.Bullock and D.Ish-Horowicz, unpublished data). Also, stem-loops from different transcripts, each of which is relatively inactive in isolation, can complement to mediate completely efficient localization when combined in the same transcript (S.L.Bullock and D.Ish-Horowicz, unpublished data). Thus, we favour the view that different transcripts share similar higher-order features, such as tertiary RNA conformations of the stems or RNA oligomers, which are recognized by the same factor(s). Multiple RNA motifs per signal and/or RNA or

protein oligomerization would lead to the formation of the multiple protein–RNA contacts that confer specificity.

RNA/BicD/Egl association appears to be a prerequisite for transport (Bullock and Ish-Horowicz, 2001). BicD is unlikely to bind RNA directly because it lacks a known RNA-interaction domain, but could hetero- or homo-oligomerize via its heptad repeat domains (Oh *et al.*, 2000) and thereby increase the numbers of protein–RNA contacts. Egl includes a domain with homology to certain 3′–5′ exonucleases and a variety of other nucleic acid-interacting proteins (Moser *et al.*, 1997), and thus might recognize RNA directly. However, its ability to recognize specific RNA sequences or structures has yet to be demonstrated.

Kinetics of transcript movement imply regulation of motor activity by RNA cargoes

Our time-lapse movies show that injected wild-type *h* transcripts can be transported in large particles, although it appears that a significant proportion of RNA is transported apically as cargoes that are too small to be detected individually. It is likely that the detectable RNA cargoes behave similar to the smaller ones because their apical accumulation closely mirrors that of the overall RNA population. Wild-type transcript particles are transported intermittently, exhibiting extensive periods of both high and low motility. Such behaviour is reminiscent of the transport by dynein of other cargoes (e.g. Shah *et al.*, 2000), including lipid droplets in the *Drosophila* blastoderm embryo (Welte *et al.*, 1998; Gross *et al.*, 2000). Non-directed movement of transcripts could result from loss of dynein processivity while transcripts remain attached to microtubules. Indeed, during these periods, transcripts do not appear to undergo significant lateral diffusion, and rarely move out of the plane of focus. However, the RNA/motor complex might detach from microtubules temporarily, large-scale diffusion being prevented by entrapment within the cytoskeletal network. Similar mechanisms could contribute to the maintenance of transcript particles at their apical destination.

A major reason for the delayed localization of weakly localizing mutant transcripts appears to be severe impairment in their ability to form active transport complexes. This is indicated by the striking delay in the onset of directional motility, as though these mutations compromise interactions of the HLE with one or more components of the localization machinery.

We had anticipated that complexes assembled by weak localizing RNAs would be as efficient as those formed on wild-type transcripts. This is clearly not the case: mutant transcripts assemble complexes that are transported at significantly reduced speed, although we currently cannot image at sufficient spatial and temporal resolution to determine whether this is due to increased detachment from microtubules or from altered motor activity on microtubules.

Mutant RNA signals might assemble complexes with different compositions of motors and/or associated factors that regulate motor activity (Gross *et al.*, 2002). Alternatively, different cargoes might dissociate more readily from the complex, or modulate the conformation or post-translational modification of motor components, for example by phosphorylation of motor components

(reviewed in Karcher *et al.*, 2002). In any case, the kinetics of transport of the three weak localizing mutants tested, as well as their assembly into active complexes, are remarkably similar. This suggests that the different changes in SL1 in these transcripts impinge upon the same regulatory process(es).

We find that localization of particles containing similar proportions of wild-type and mutant RNAs is indistinguishable from that observed when the weak localizing mutants are injected alone. This argues against a mechanism in which individual transcripts in a particle act independently to determine the overall efficiency of transport, for example by separately recruiting or activating the motor(s) or accessory factor(s). Characterization of complexes assembled on the HLE should lead to a greater understanding of the molecular basis of motor regulation.

Despite the impaired transport of mixed-transcript particles, the overall localization of the wild-type transcript population is efficient, indicating that different mRNAs localize somewhat independently. This apparent paradox is most easily resolved if a proportion of the *h* RNA localizes efficiently as small particles below our detection limit. These might be less susceptible to dominant-negative effects of mutant cargoes if they contain predominantly wild-type transcripts. Indeed, it is possible that individual transcript molecules can be transported.

Our results provide evidence that subtle changes in the cargo can modulate the efficiency of motor transport. Cargo/motor interactions would not only help ensure that motors are not activated until they are associated with cargo (Friedman and Vale, 1999), but could also allow precise modulation of cargo distribution by varying transport speed.

Materials and methods

Details of plasmid construction and cloning of *h* 3′UTRs from *Drosophila* species can be found in the Supplementary data.

Fluorescent mRNA injection

Wild-type *D.melanogaster* were of the Ore-R strain. Synthesis and injection of mRNAs labelled with Alexa 488-UTP (Molecular Probes), Cy3-UTP or Cy5-UTP (Perkin Elmer), fixation of embryos and immunodetection of Egl and BicD were as described previously (Bullock and Ish-Horowicz, 2001). Typically, 20–50 embryos were injected with each transcript and fixed 5 min after injection of the last embryo (~8 min after injection of the first). A total concentration of 250 ng/μl for each mRNA was injected routinely, except for experiments assaying the recruitment of Egl and BicD and for time-lapse analysis, when the mRNA concentration was 1 μg/μl. For time-lapse analysis of individual constructs, Alexa 488-labelled RNAs were used. For movies of co-injected *h* and 1328^{e33}A→U, Cy3- and Alexa488-labelled RNAs were mixed (final concentration of 500 ng/μl each) and injected. Consistent results were obtained with different RNA preparations, fluorochromes and days of injection.

Imaging and analysis of particle motility

Fixed embryos were imaged with a Zeiss LSM 510 laser scanning confocal microscope. For time-lapse imaging, live embryos were mounted on a No. 0 coverslip (Chance Propper) with heptane glue and viewed under 10S halocarbon oil with an UApo 340 40×/1.35NA oil immersion iris objective on an Olympus IX70 inverted microscope. Embryos were visualized with an UltraVIEW LCI confocal scanner using an Ultrapix (Perkin Elmer) or Orca ER (Hamamatsu) camera at a lapse interval of 1.18 s (or 4.6 s to confirm that *h* and 1328^{e33}A→U transcripts are co-transported). Image acquisition from a chosen focal plane started 60 s after injection.

Particles were tracked manually using Motion Analysis software (Kinetic Imaging), beginning when they first displayed characteristics of active transport (i.e. at least three successive displacements in a persistent direction). We tracked all such particles in the observation field. Tracking ended when particles reached the apical cytoplasm or, more rarely, moved out of the plane of focus. In separate experiments addressing motility of particles in the apical cytoplasm, we tracked 10 particles in at least two different embryos for >30 s. All tracks were exported as sequences of (*x*, *y*) co-ordinates and analysed using custom software in Mathematica 4.0 (Wolfram Research).

A particle trajectory consists of consecutive positions (*x_i*, *y_i*), where *i* is frame number between 1 and *N*. Displacements were calculated with two different time resolutions: one lapse interval,

$$1.18 \text{ s } [d_{1i} = \sqrt{(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2}, i \text{ is between } 1 \text{ and } N - 1]$$

and two lapse intervals,

$$2.36 \text{ s } [d_{2i} = \sqrt{(x_{i+2} - x_i)^2 + (y_{i+2} - y_i)^2}, i \text{ is between } 1 \text{ and } N - 2].$$

Persistence *p_i* was evaluated as *p_i* = *d_{2i}*/(*d_{1i}* + *d_{1i+1}*), where *i* is between 1 and *N* - 2. Persistence defined in this way gives numbers between 0 and 1. A value of 0 is for the lowest persistence when in two steps the particle remains static or returns to its original position and persistence of 1 is for movement in a straight line. Transport was defined as directed when *p_i* > 0.5, *d_{1i}* > 0.01 μm and *d_{1i+1}* > 0.01 μm. This definition led to a well-separated bimodal distribution and tallied well with our manual classification of periods of directed and non-directed motion of individual tracks.

For each particle, we calculated the mean of all displacements *d_{2i}* and the mean of directed displacements *d_{2i}*. These values were expressed as speed in μm/s and used to calculate the mean, SEM and maximum velocity for all particles of a given transcript. To verify that the maximum was a robust measure, it was also evaluated with lower time resolution and similar values were obtained. Frequencies of directed motion were also evaluated for each transcript. Significances of differences between transcripts were tested in Mathematica 4.0 using ANOVA with a nested unbalanced model (Milliken and Johnson, 1992).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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