

Cis-acting determinants of asymmetric, cytoplasmic RNA transport

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ABSTRACT

Asymmetric subcellular distribution of RNA is used by many organisms to establish cell polarity, differences in cell fate, or to sequester protein activity. Accurate localization of RNA requires specific sequence and/or structural elements in the localized RNA, as well as proteins that recognize these elements and link the RNA to the appropriate molecular motors. Recent advances in biochemistry, molecular biology, and cell imaging have enabled the identification of many RNA localization elements, or “zipcodes,” from a variety of systems. This review focuses on the mechanisms by which various zipcodes direct RNA transport and on the known sequence/structural requirements for their recognition by transport complexes. Computational and experimental methods for predicting and identifying zipcodes are also discussed.

Keywords: RNA zipcode; cytoplasmic RNA transport; localized RNA

INTRODUCTION

Multiple mechanisms exist for ensuring that proteins are produced in the appropriate place and time within a cell or organism. Many of these regulatory processes act at the post-transcriptional level. Localization of mRNA is commonly employed to target protein products to specific regions within a cell. Some mRNAs are localized to appropriate subcellular structures at the same time they are being translated, and it is the emerging nascent peptide that directs the localization of both the ribosome and the transcript. For example, transcripts encoding secreted proteins are localized to the endoplasmic reticulum (ER) by recruitment of the nascent signal peptide (Nicchitta 2002). In *Bacillus subtilis*, the transmembrane domains of the glucose transporter IICB target the ribosome–mRNA–protein complex to the cell membrane (Kawamoto et al. 2005). In other cases, such as the localization of some

transcripts to mitochondria in yeast, transport is driven by sequences in the RNA itself (Corral-Debrinski et al. 2000). Asymmetric subcellular distribution of RNA is critical for proper embryo development, for establishing differences in cell fate, and for neuronal function (Chartrand et al. 2001). Unlike the examples of RNA localization to the ER, asymmetric cytoplasmic RNA localization is generally a motor-driven process and, like the transport of RNAs to mitochondria, is commonly directed by *cis*-acting elements. The RNA is recognized by RNA-binding protein(s) and the resulting RNA–protein complex, or mRNP, is linked either directly or indirectly to molecular motors that transport the mRNP to its final destination (Oleynikov and Singer 1998). Following transport, the mRNA is anchored at its site of translation; additional mechanisms often exist to ensure that the mRNA is not prematurely translated during transport. Examples of this process occur in organisms from yeast to mammals (Tekotte and Davis 2002).

“Zipcodes” are *cis*-acting motifs that direct mRNAs for transport to appropriate locations within a cell or organism (Kislauskis and Singer 1992). Zipcodes range in length from a few nucleotides to over 1 kb; however, it is possible that some of the longer zipcodes have not been reduced to their minimally sufficient lengths, making it difficult to identify key RNA determinants of transport. It is believed that zipcodes serve as binding sites for proteins that form complexes with molecular motors, thereby linking the RNA to the cellular transport machinery (Mowry 1996). In yeast,

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the interactions between the RNA-binding species, protein linker, and motor have been defined (Bohl et al. 2000; Long et al. 2000; Takizawa and Vale 2000). In other systems, the involvement of motors in the transport process has been established (Brendza et al. 2000; Januschke et al. 2002; Betley et al. 2004; Yoon and Mowry 2004), but the linkage of RNA-binding proteins and accessory factors to motors remains uncharacterized. Often, translation inhibitors are associated with the transport mRNP complex to ensure that the mRNA is not prematurely translated en route to its final destination. Translational control has been reviewed elsewhere (e.g., Bashirullah et al. 1998; Johnstone and Lasko 2001; Kindler et al. 2005; Sossin and Desgroseillers 2006) and will not be discussed here. Additional RNA sequences may be required to promote the assembly of a localization-competent RNP (Czaplinski and Mattaj 2006).

An essential feature of zipcodes is that they can direct localization independently of the adjacent RNA sequence: fusing a zipcode to a reporter RNA results in a subcellular distribution of the reporter similar to that observed for the native mRNA. Although zipcodes are usually located in 3' untranslated regions (UTRs) of transported messages (e.g., Mowry and Melton 1992; Kim-Ha et al. 1993; Gavis et al. 1996a; Zhou and King 1996b; Deshler et al. 1997; Macdonald and Kerr 1997; Chan et al. 1999), in some instances they can mediate localization when placed at the 5' end or even in the middle of a reporter RNA (Cohen et al. 2005), although not all zipcodes are position independent (Kislauskis et al. 1994). Zipcodes in bud-localized yeast RNAs, in contrast to transported RNAs in metazoans, are generally found in coding regions (Shepard et al. 2003), but can function efficiently when located in 3' UTRs of reporter transcripts (e.g., Chartrand et al. 1999; Gonzalez et al. 1999; Hachet and Ephrussi 2004).

IDENTIFICATION OF ZIPCODES

Identifying zipcodes has been problematic for several reasons. In many cases, the essential RNA-binding protein(s) of the transport mRNP is not known a priori, since transported RNAs are often associated with large, multi-protein complexes. Therefore, it is often not possible to screen for zipcodes based on protein-binding ability (e.g., to select for RNAs that bind an immobilized protein); once mapped, however, zipcode sequences have been successfully used to identify cognate RNA-binding proteins (e.g., Gautreau et al. 1997; Hoek et al. 1998; Cote et al. 1999; Bergsten et al. 2001). Second, many zipcodes are recognized by the transport machinery on the basis of both sequence and structure. For example, the *bicoid* localization element contains one helical region, in which only the secondary structure but not sequence is important, as well as a region in which base identities are essential for transport (Macdonald and Kerr 1998). Furthermore, reporter sequences used to test putative zipcodes for localization activity could induce

the zipcode to adopt a different structure than it does in the native RNA, thus producing a false negative result. Improper folding of zipcodes out of context may be the reason that many have not been minimized beyond several hundred nucleotides in length. In fact, differences in reporter constructs have been invoked (Huang et al. 2003) to explain why some groups observed that the full-length 3' UTR of α CamKII localized to dendrites (Mayford et al. 1996; Blichenberg et al. 2001) and another group found that it did not (Mori et al. 2000). Thus, efforts to detect zipcodes by motif-finding algorithms may be hampered both by the inherent degeneracy of the sequence and by the availability of minimally sufficient, validated zipcodes. Third, differences in criteria for determining "localized" RNAs can affect which sequences are identified as zipcodes. For example, Ferrandon et al. (1994) reported that the dimerization domain III of the *bicoid* 3' UTR is sufficient for embryonic localization based on its ability to recruit Staufen and localize to astral microtubules (a localization pattern not observed for full-length *bicoid*). In contrast, when Snee et al. (2005) mapped the regions required to achieve a wild-type (WT) *bicoid* localization pattern at the anterior of the embryo, stem-loops IV–V were required in addition to domain III. Similarly, the discrepancies in localization elements identified in α CamKII have also been attributed to localization scoring criteria (Huang et al. 2003). Two groups counted RNAs as "dendritically localized" if they were at a distance of more than one soma diameter from the cell body (Mori et al. 2000; Blichenberg et al. 2001), whereas another group scored RNAs located along the lengths of dendrites (Huang et al. 2003). Fourth, many RNAs are localized by multistep processes, with each step being governed by distinct zipcodes and potentially coupled to the preceding and/or subsequent step (see below). Therefore, multiple elements are required together to effect localization, precluding the identification of minimal subelements.

The most common method for identifying zipcodes involves monitoring the subcellular distributions of fragments derived from the localized RNA, either on their own or fused to reporter transcripts. Akin to transcriptional promoter bashing, this brute force approach identifies sequences that are sufficient for transport. Alternately, deletion of regions within the native RNA that impair localization can be used to identify essential sequences. In cases where the RNA-binding protein is known, putative zipcode sequences can be isolated rapidly and efficiently on the basis of their binding ability (Jambhekar et al. 2005); however, as noted above, the RNA-binding species in many localization pathways are not known.

Many methods have been developed for monitoring RNA distribution in live or fixed cells. Early studies in *Drosophila* were conducted by detecting transcripts of interest in sections of cells or embryos (Macdonald and Struhl 1988). Traditionally, RNA distribution has been

visualized in fixed cells by fluorescence in situ hybridization (FISH) to native or reporter RNAs. RNA transport and localization can be visualized in live cells by injecting fluorescently labeled RNA; while this technique works well in some oocytes and embryos, it is not feasible for smaller cells such as yeast and bacteria. Furthermore, because RNAs can be “marked” for cytoplasmic transport early on in the nucleus (Kruse et al. 2002; Kress et al. 2004), it is likely that RNAs injected in the cytoplasm may not recruit the full complement of proteins necessary for transport. And in some cases, the fluorescent tag on the RNA can compromise its structural integrity (Wilkie et al. 2001). Other techniques developed more recently allow visualization of fully processed mRNA in multiple cell types. A reporter RNA containing MS2 stem-loop aptamers can be tracked by coexpressing an MS2-coat protein–GFP fusion (Fig. 1A); this strategy is effective in both yeast and mammalian cells (Bertrand et al. 1998; Fusco et al. 2003), but the MS2 aptamers and/or bound MS2-coat protein may interfere with localization of some RNAs. Molecular beacons can also be used to visualize native RNA in live cells (Fig. 1B; Bratu et al. 2003). This technique involves injecting an oligonucleotide probe (“molecular beacon”) complementary to the native RNA. Self-complementary sequences at the 5' and 3' ends of the probe induce it to fold in a hairpin; a fluorophore is coupled to one end and a quencher at the other. In the folded state, the 5' and 3' ends are in close proximity and all fluorescence is quenched. Upon binding to the target RNA, the hairpin probe unfolds and the quencher is removed from the vicinity of the fluorophore, resulting in a fluorescent signal. A similar approach employs two molecular beacons that anneal to adjacent regions of the target RNA. One beacon is conjugated to a donor fluorophore and the other to an acceptor, resulting in a FRET signal upon specific binding of both beacons to their target (Santangelo et al. 2004). If the molecular beacons overlap with zipcodes, however, they could mask recognition by the transport machinery and preclude identification of bona fide zipcodes. In fact, masking of zipcodes by hybridization to exogenous oligonucleotides has been used to identify zipcodes in loss-of-function assays (Kislauskis et al. 1994). Alternately, binding of the transport machinery to localized transcripts may

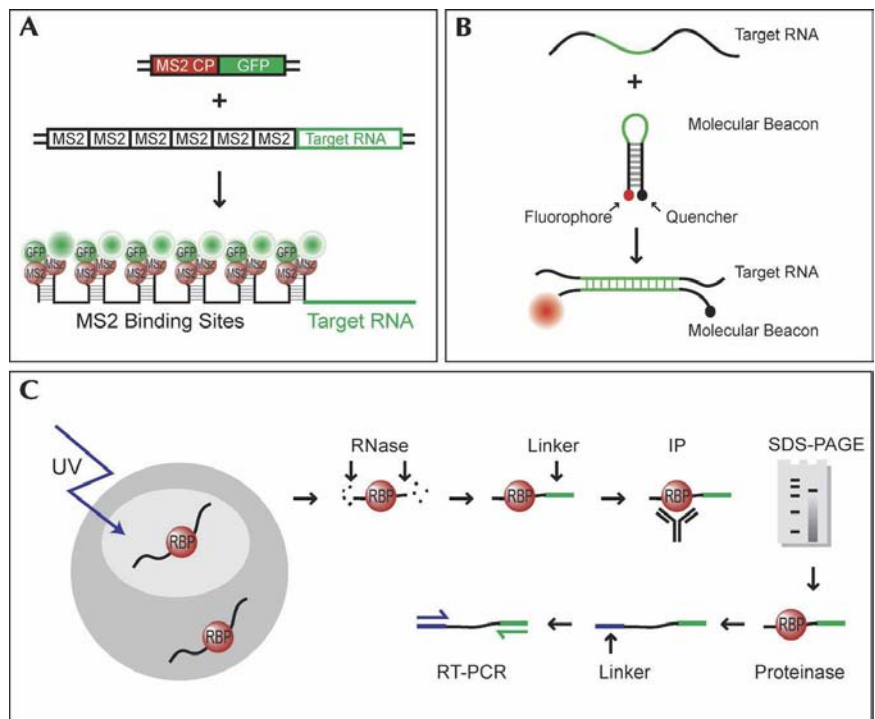


FIGURE 1. Schematics of zipcode identification strategies. (A) *in vivo* GFP-RNA tagging (after Bertrand et al. 1998). The RNA of interest is expressed fused to a reporter containing six tandem repeats of the MS2 stem-loop sequence. An MS2-coat protein–GFP fusion is coexpressed, resulting in a GFP-marked RNA. (B) Molecular beacons (after Bratu et al. 2003). A hairpin oligonucleotide containing sequences complementary to the target RNA is conjugated to a fluorophore at one end and a quencher at the other. In the hairpin conformation, the fluorophore is quenched. Upon injection of the molecular beacon into cells and subsequent hybridization to the target RNA, the quencher is removed from the proximity of the fluorophore and the RNA can be tracked based on a signal from the fluorophore. (C) CLIP (after Ule et al. 2005). RNA–protein complexes are cross-linked with UV, isolated, and treated with RNase. Protein-bound RNA regions are protected from digestion. A linker is ligated to the 3' end, and intact complexes are isolated by immunoprecipitation of the RNA-binding protein, followed by SDS-PAGE purification. The protein is then removed by digestion. A second linker is ligated to the 5' end and the isolated RNA is amplified by reverse transcription and PCR.

prevent hybridization of the RNA to the beacon probe, resulting in a loss of signal.

The recently developed cross-linking and immunoprecipitation (CLIP) technique (Ule et al. 2003) also promises to be effective at isolating zipcode sequences (Fig. 1C). In this method, nucleic acids and proteins are first cross-linked, followed by cell lysis and RNase treatment of nucleic acids. The mRNP protein of interest is immunoprecipitated, and the bound RNA is isolated, cloned, and sequenced (Ule et al. 2005). This method has the advantage of isolating RNA–protein complexes under physiological conditions. Furthermore, because mRNP complexes are cross-linked prior to purification, the identity of the RNA-binding protein does not need to be known; in principle, any component of the mRNP can be used for immunoprecipitation. Therefore, this technique could be especially powerful for isolating zipcodes from systems in which the RNA-binding protein remains undefined; to

date, however, no zipcodes have been identified by this method.

ZIPCODES ACT IN CONCERT TO DIRECT RNA LOCALIZATION

Localization of RNAs often occurs by a multistep process. A localization-competent RNP must first be assembled on the RNA, after which the complex is transported to its final destination. For a given RNA, transport can occur in multiple spatially or temporally distinct steps or can be affected by multiple redundant mechanisms. In many cases, the RNA is anchored at its final destination. Each of these steps may be directed by dedicated *cis*-acting signals in the transcript.

Some RNAs contain all information necessary for successful transport in a single zipcode. The non-protein-coding, dendritically localized BC1 RNA contains a 62 nucleotide (nt) zipcode at its 5' end (Muslimov et al. 1997), which is unlikely to contain multiple, independently functioning subelements due to its short length. This zipcode may directly recruit the machinery responsible for transporting it in a single microtubule-dependent step (Cristofanilli et al. 2006), and the RNA may not have *cis*-acting sequences for transport by redundant mechanisms. Another dendritically localized transcript, the MAP2 mRNA, contains a 640 nt element in its 3' UTR that is necessary and sufficient for transport (Blichenberg et al. 1999). Because this element is large and is predicted to contain multiple structural domains, it is possible that distinct subelements within the zipcode mediate individual steps of the localization process (see Table 1).

Unlike BC1, many RNAs contain multiple sequence/structural motifs essential for proper localization. In oligodendrocytes, two distinct elements in the MBP RNA mediate its transport to and retention at the myelin compartment. An 11 nt element, A2RE, serves as a binding site for hnRNP A2 (Hoek et al. 1998) and is necessary and sufficient for transport (Munro et al. 1999). This element is found in other transported RNAs but not in isoforms of transcripts that are unlocalized (Ainger et al. 1997), suggesting that it might be a key determinant for targeting RNAs for transport. A second, more complex, element over 1 kb in length is essential for proper localization of a protein-coding reporter RNA following transport, and may be involved in RNA anchoring (Ainger et al. 1997).

Like MBP RNA, the *Drosophila oskar* (*osk*) and *gurken* (*grk*) mRNAs are localized in multiple, nonoverlapping steps with earlier localization events being required for later ones. *osk* contains three regions that direct three steps of the localization process (Kim-Ha et al. 1993): (1) movement of the RNA from nurse cells into the oocyte; (2) accumulation at the anterior margin; and (3) localization at the posterior end of the oocyte. *Cis*-acting determinants directing each step have been mapped in the 3' UTR, and it

was hypothesized that different combinations of *cis* elements could direct RNAs to different final destinations (Kim-Ha et al. 1993). The three steps of *osk* localization appear nonoverlapping: elimination of early events by mutation of the corresponding zipcode generally precludes later steps of the localization process (Kim-Ha et al. 1993).

gurken mRNA displays a two-step localization pattern in oocytes that is governed by two zipcodes (Saunders and Cohen 1999; Thio et al. 2000). During stages 1–6, *grk* localizes to the posterior of oocytes. The determinants of this localization activity are inferred to lie within the first 35 bases of the protein-coding region (termed GLE1), but this sequence has not been tested directly for zipcode activity (Saunders and Cohen 1999). During stage 8, *grk* relocates to the anterodorsal corner of the oocyte, and this localization is mediated by 64 nt downstream of GLE1. The predicted secondary structure of this element is similar to that of a zipcode derived from the *I* factor transcript, an LTR RNA whose localization during stages 8–9 parallels that of *grk* (Van De Bor et al. 2005). While Van de Bor and colleagues did not report a role for the *grk* 3' UTR in directing localization (Van De Bor et al. 2005), two other studies reported that the 3' UTR was necessary for the final stages of anterodorsal accumulation (Saunders and Cohen 1999; Thio et al. 2000). This discrepancy was attributed to differences in reporters used for assaying localization (Van De Bor et al. 2005). All three studies agreed that the 3' UTR of *grk*, unlike that of several other transcripts localized in *Drosophila* oocytes, has no localization activity on its own (Saunders and Cohen 1999; Thio et al. 2000; Van De Bor et al. 2005).

In contrast to the above transcripts, which are transported in multiple, nonoverlapping steps, some RNAs contain multiple localization elements that mediate transport via partially redundant mechanisms. For example, two fragments within a 280 nt zipcode in the 3' UTR of *orb* can independently mediate its localization to the oocyte posterior during early stages, but both are required together for accumulation at the anterior end of *Drosophila* oocytes during late stages (Lantz and Schedl 1994).

Localization of *bicoid* (*bcd*) mRNA to the anterior end of *Drosophila* oocytes also occurs in distinct steps, termed event A (which acts during early phases of oogenesis, stages 4–5) and event B (which occurs later, after stage 6). Both events are directed by 650 nt in the 3' UTR (Macdonald and Struhl 1988). The entire UTR is nearly 900 nt in length and folds into five distinct domains (Brunel and Ehresmann 2004). Event A is mediated by stem-loops IV–V in the 3' UTR (Macdonald and Kerr 1997), or artificially by a tandem repeat of a 53 nt sequence (called BLE1) within stem-loop V (Macdonald et al. 1993), which binds transport complex components Ex1 and Exu (Macdonald et al. 1995). A single-base mutation in stem-loop V (G4496U) eliminates event A, and *bcd* accumulation at the anterior of the oocyte occurs later via event B (Macdonald and Kerr

TABLE 1. Summary of zipcodes in localized transcripts discussed in the text

Organism	RNA	Zipcode region	Important features	Subcellular destination	Necessary?/sufficient?	Cognate binding protein	References	
<i>Drosophila</i>	<i>bicoid</i>	3' UTR (650 nt)		Oocyte and embryo anterior	Yes/yes		Macdonald and Struhl (1988)	
		Stem-loop IV/V	Guanosine 4496, other sequence and structural elements	Oocyte anterior	For event A only/yes		Macdonald and Kerr (1997, 1998)	
		BLE1 (54 nt from stem-loop V)	Guanosine 4496, other sequence and structural elements	Oocyte anterior	For event A only/yes, X2	Exl, Exu	Macdonald et al. (1993) Macdonald and Kerr (1998)	
		Domain III	Dimerization ability	Embryo anterior	Yes/no	Staufen	Ferrandon et al. (1997) Snee et al. (2005) Kim-Ha et al. (1993)	
	<i>oskar</i>	3' UTR		Oocyte anterior, then posterior	Yes/no			
		SacI/PstI region (~150 nt)		Oocyte	Yes/not tested		Kim-Ha et al. (1993)	
		EcoRI/PvuII region (~150 nt) and PstI/EcoRI region (~50 nt)		Transport out of anterior margin	Each is necessary/not tested		Kim-Ha et al. (1993)	
	<i>gurken</i>	StyI/DraI region (~350 nt) 5' end of ORF		Oocyte posterior	Yes/not tested		Kim-Ha et al. (1993)	
		GLE1 (35 nt)		Oocyte posterior, then dorsoanterior	Yes/yes		Saunders and Cohen (1999)	
		GLS (64 nt stem-loop)		Oocyte posterior	Yes/not tested		Saunders and Cohen (1999)	
<i>I factor orb</i>		ILS (58 nt stem-loop at 5' end) 3' UTR		Oocyte anterior	Yes/yes		Van De Bor et al. (2005)	
				Oocyte posterior, then anterior	Yes/yes		Van De Bor et al. (2005)	
		280 nt region		Oocyte posterior, then anterior	Yes/yes		Lantz and Schedl (1994)	
	<i>nanos</i>	3 UTR		Oocyte posterior	Yes/yes		Gavis and Lehmann (1992) Gavis et al. (1996a)	
		Four partly redundant regions (+1 to +4) +2'ME (41 nt)		Oocyte posterior	Not tested/each localizes weakly		Gavis et al. (1996a)	
		3' UTR		Oocyte anterior	No/yes, X3		Bergsten et al. (2001)	
	<i>K10</i>	TLS (44 nt stem-loop)	Minor groove structure and some primary sequence	Oocyte anterior	Yes/yes		Cheung et al. (1992) Serano and Cohen (1995)	
	<i>Rat</i>	<i>MAP2</i>	3' UTR		Dendrites	Yes/yes		Blichenberg et al. (1999)
			640 nt region		Dendrites	Yes/yes		Blichenberg et al. (1999)
		<i>BC1</i>	5' end		Dendrites	Not tested/yes		Muslimov et al. (1997)
		62 nt stem-loop		Dendrites	Not tested/yes		Muslimov et al. (1997)	
α CaMKII		3' UTR		Dendrites	Yes/yes		Mayford et al. (1996)	
	94 nt after stop codon	30 nt primary sequence	Dendrites	Yes/yes		Mori et al. (2000)		
	1200 nt at 3' end		Dendrites	Not tested/yes		Blichenberg et al. (2001)		

(continued)

TABLE 1. (Continued)

Organism	RNA	Zipcode region	Important features	Subcellular destination	Necessary?/sufficient?	Cognate binding protein	References
	neurogranin	CPE (170 nt region)	30 nt sequence in 3' UTR homologous to α CamKII	Dendrites	Not tested/yes	CPEB	Huang et al. (2003)
		Not mapped		Dendrites	Yes/not tested		Mori et al. (2000)
	MBP	3' UTR	Primary sequence	Myelin sheath	Yes/yes	hnRNP A2	Ainger et al. (1997)
		A2RE (11 nt)		Oligodendrocyte processes	Yes/yes		Hoek et al. (1998)
	Vasopressin	1 kb region	Guanosine (possibly presence of stop codon)	Myelin sheath	Yes (for protein-coding reporters)/not tested		Ainger et al. (1997)
		ORF fragment		Dendrites	Not tested/yes		Prakash et al. (1997)
	tau	ORF + 3' UTR fragment	U-rich region	Dendrites	Not tested/yes		Prakash et al. (1997)
		ORF		Axons	Yes/not tested		Mohr et al. (1995)
<i>Xenopus</i>	<i>Xcat2</i>	3' UTR	U-rich region	Axons	Yes/yes	huD	Behar et al. (1995)
		280 nt fragment		Axons	Yes/yes		Aranda-Abreu et al. (1999)
	<i>fatg</i>	250 nt MCLE (via METRO pathway)	Vegetal pole	Vegetal pole	Yes/yes		Aronov et al. (2001)
		164 nt GGLE (via METRO pathway)		Vegetal pole via mitochondrial cloud	Yes/yes		Zhou and King (1996a,b)
	<i>Vg1</i>	150 nt downstream of stop codon + 120 nt at 3' end (via late pathway)	U-rich region	Germinal granules	Yes/yes		Zhou and King (1996a)
		3' UTR		Vegetal pole	Yes/yes		Kloc et al. (2000)
	<i>Xvelo</i>	75 nt stem-loop at 3' end	Stem and tail sequences and secondary structure	Vegetal pole	Yes/yes	VgRBP/Vera	Chan et al. (1999)
		100 nt at 5' end		Vegetal pole	Not tested/yes		Chan et al. (1999)
	<i>Xisirt</i>	P11 repeat	VM1, E2, FVLE1 sites	Vegetal pole	Not tested/yes	hnRNP I	Chan et al. (1999)
		137 nt region		Vegetal pole	Not tested/yes		Mowry and Melton (1992)
<i>Chicken</i>	β -actin	5' UTR	Stem and tail sequences and secondary structure	Vegetal pole	Yes/yes		Deshler et al. (1997)
		300 nt region		Vegetal pole	Yes/no		Deshler et al. (1997, 1998)
	<i>Xisirt</i>	3' UTR	Stem and tail sequences and secondary structure	Vegetal pole	Yes/yes		Gautreau et al. (1997)
		3' UTR		Vegetal pole	Not tested/yes		Claussen and Pieler (2004)
	<i>XNIF</i>	3' UTR	Stem and tail sequences and secondary structure	Vegetal pole	Not tested/yes		Claussen and Pieler (2004)
		3' UTR		Vegetal pole	Not tested/yes		Claussen and Pieler (2004)
	β -actin	3' UTR	Stem and tail sequences and secondary structure	Vegetal pole	Not tested/yes		Claussen and Pieler (2004)
		3' UTR		Vegetal pole	Not tested/yes		Allen et al. (2003)
	<i>XNIF</i>	3' UTR	Stem and tail sequences and secondary structure	Vegetal pole	Not tested/yes		Allen et al. (2003)
		3' UTR		Vegetal pole	Not tested/yes		Claussen et al. (2004)
	β -actin	3' UTR	Stem and tail sequences and secondary structure	Vegetal pole	Yes/yes		Claussen et al. (2004)
		3' UTR		Vegetal pole	Yes/yes		Kislauskis et al. (1994)
	<i>XNIF</i>	3' UTR	Stem and tail sequences and secondary structure	Fibroblast leading edge, nerve growth cones	Yes/yes		Bassell et al. (1998)
		3' UTR		Fibroblast leading edge, nerve growth cones	Yes/yes		

(continued)

TABLE 1. (Continued)

Organism	RNA	Zipcode region	Important features	Subcellular destination	Necessary?/sufficient?	Cognate binding protein	References
		54 nt zipcode	GGACT AATGC ACACCC	Fibroblast leading edge	Not tested/yes Not tested/in tandem repeat with $\times 1$ AATGC Not tested/in tandem repeat with $\times 1$ GGACT		Kislauskis et al. (1994) Kislauskis et al. (1994) Kislauskis et al. (1994)
Yeast	ASH1 and others	43 nt zipcode ORFs	Stem-loops with single-stranded CG and other essential sequences	Fibroblast leading edge Bud tips	Yes/not tested Not tested/yes (weak) Yes (tested only for ASH1)/yes	ZBP1 She2/3	Ross et al. (1997) Kislauskis et al. (1994) Chartrand et al. (1999) Gonzalez et al. (1999) Jambhekar et al. (2005) Olivier et al. (2005)

The organism, zipcode region, and subelements, as well as features essential for zipcode function are indicated. "Necessary" indicates that deletion of the zipcode in the context of surrounding native sequences abolishes localization. For transcripts containing multiple zipcodes, a zipcode is considered "not necessary" only if its deletion has been shown not to affect localization. No assumptions are made about zipcode redundancy in these cases. "Sufficient" indicates that the region localizes in the absence of surrounding native sequences. " $\times 2$ " or " $\times 3$ " indicates that the region functions when present as a tandem repeat of two or three copies, respectively.

1997). *Cis*-acting determinants of event B have not been isolated, but some *trans*-acting factors necessary for localization during and after event B (e.g., Swallow, Exuperantia, and Staufen) have been identified (Berleth et al. 1988; Stephenson et al. 1988; St Johnston et al. 1991; Macdonald and Kerr 1997).

Like events A and B in *bcd* localization, two pathways—message transport organizer (METRO) and the late pathway—effect transport of RNAs to the vegetal pole of *Xenopus* oocytes. Recently, the two pathways were shown to have some transport complex components in common, indicating a partial overlap in function (Claussen et al. 2004; Choo et al. 2005); accordingly, several RNAs are substrates of both pathways (see below), suggesting functional redundancy. The METRO pathway acts during early stages of oogenesis and transports RNAs via the mitochondrial cloud. Substrates of this pathway include *Xcat-2* (Forristall et al. 1995) and *Xlsirt* (Kloc et al. 1993) RNAs. During transport via the METRO pathway, *Xcat-2* localizes to germinal granules within the mitochondrial cloud (Kloc et al. 1998), and, like the MBP 3' UTR, it contains distinct elements governing its localization to appropriate cellular structures. A 250 nt mitochondrial cloud localization element (MCLE) at the 5' end of the 3' UTR directs the RNA to the mitochondrial cloud (Zhou and King 1996a), and specific localization to the germinal granules is mediated by a downstream 164 nt germinal granule localization element (GGLE) (Kloc et al. 2000). *Xcat-2* can also localize in stage IV embryos via the late pathway, and the sequences mediating localization by this pathway map to the 5' and 3' ends of the 3' UTR (Zhou and King 1996b). These determinants overlap partially with both the MCLE and the GGLE, yet the exact sequences required for localization via the late pathway are distinct from those required for the METRO pathway. Another vegetally localized mRNA, *fatvg*, also localizes by both the METRO and late pathways and contains multiple localization elements in its 3' UTR (Chan et al. 1999, 2001). A 25 nt sequence from the 5' end of the UTR (the *fatvg* localization element, FVLE1) is sufficient for localizing RNA via the late pathway (Chan et al. 1999), and it is likely that some of the other zipcodes mediate localization by the METRO pathway. Like *Xenopus*, ascidians also employ two different pathways of localizing RNAs in embryos, with each pathway requiring distinct zipcode elements (Sasakura and Makabe 2002).

The studies in *Drosophila* and *Xenopus* suggest that zipcodes are modular and that different combinations of elements can direct different localization programs, but this principle has not been extensively tested. Differentially localized transcripts rarely contain shared elements, indicating that zipcodes are not used in a modular manner in vivo. Few attempts have been made at engineering RNAs with novel subcellular destinations by incorporating appropriate zipcodes. In one case, however, addition of the GGLE to the *Xlsirt* MCLE did localize the hybrid RNA to germinal granules (Kloc et al. 2000). Identification of additional

zipcodes in localized transcripts, as well as efforts to engineer localized RNAs, will provide more information about the modularity of zipcode function.

ZIPCODE ELEMENTS ACT SYNERGISTICALLY TO TARGET RNAS TO THE TRANSPORT MACHINERY

Not only can multiple elements mediate multiple steps in the localization process, elements can also act synergistically to promote individual steps. In some cases, multimers of a single motif promote localization synergistically; and in other cases, different motifs cooperate to ensure localization. Many transcripts employ a combination of both strategies in order to ensure efficient and specific transport.

The *Vg1* localization element, which encompasses >300 nt in the *Vg1* 3' UTR (Mowry and Melton 1992; Deshler et al. 1998) and is responsible for localizing the RNA to the vegetal pole of *Xenopus* embryos, contains four sequence elements, E1–E4, in two to four copies each (Deshler et al. 1997) as well as a VM1 element in three copies (Gautreau et al. 1997). Deletion of all copies of E1, E2, E3, or E4 compromises localization efficiency, with E2 deletions showing the strongest defect. Element E2 binds Vera/VgRBP, an endoplasmic reticulum RNA-binding protein that is proposed to link *Vg1* RNA to the ER for transport (Deshler et al. 1997, 1998). Recently, bud localization of *ASH1* mRNA in yeast was also shown to require association of the transport complex with endoplasmic reticulum, suggesting that this mechanism of transport may be conserved between species (Schmid et al. 2006). However, because the E2 elements in the *fatvg* 3' UTR are dispensable for transport, it seems that this element alone is not sufficient for vegetal localization in *Xenopus* oocytes (Chan et al. 1999). The VM1 element is a binding site for hnRNP I, and a fragment containing two copies of the VM1 element can direct localization when present as a tandem repeat (Gautreau et al. 1997). However, a native cluster of five VM1 elements in the 3' UTR of *Xenopus borealis Vg1* cannot support localization (Lewis et al. 2004), suggesting that the sequence or structural context of the elements either affects recognition by hnRNP I or is necessary for other events following hnRNP I binding. It has been proposed that the *Vg1* zipcode function requires clusters of VM1 and E2 sites together (Lewis et al. 2004).

Nanos RNA contains four elements (+1 to +4) in its 3' UTR, each of which localizes weakly to the posterior of *Drosophila* oocytes; in concert, the four elements confer full localization ability (Gavis et al. 1996a). A 41 nt region within the +2' element (termed +2'ME) shows high-sequence conservation in *Drosophila melanogaster* and *Drosophila virilis* and is bound by a 75 kD protein of unknown function (Bergsten et al. 2001). Three tandem copies of +2'ME localize as efficiently as the entire +2' element, which is reminiscent of the localization of *bicoid* RNA mediated by two tandem copies of BLE1.

Surprisingly, when localization-impaired versions of the +2' ME *nanos* localization element are combined with the WT +1 element, the composite RNA localizes less efficiently than the +1 element alone, indicating that mutations in the +2' ME region impair the activity of the +1 element. This result led the authors to suggest that long-range interactions between localization elements govern transport of *nanos* RNA (Bergsten et al. 2001), but the mechanisms by which these interactions occur and are detected by the cellular transport machinery remain unknown.

A 54 nt zipcode in the 3' UTR of chicken β -actin mRNA also contains multiple motifs that direct localization synergistically to the leading edge of chicken fibroblasts (Kislauskis et al. 1994). Two motifs, GGACT and AATGC, are found in both the 54 nt zipcode and in a separate 43 nt region that shows weak localization ability. Although each motif alone (in the absence of most of the other zipcode sequences) localizes poorly, synthetic constructs containing tandem repeats of one motif along with one copy of the other show enhanced localization ability (Kislauskis et al. 1994). In addition, an AC-rich region between the two motifs of the 54 nt zipcode is essential for its activity (Kislauskis et al. 1997). Two ACACCC sequences in this region each bind to ZBP1, a protein that contains 4 KH domains and bears homology with hnRNP proteins (Ross et al. 1997) and is also involved in RNA transport (Farina et al. 2003). The stronger of the two β -actin binding sites is predicted to be single stranded (Ross et al. 1997). The same sequence motif occurs in the 3' UTR of α_3 integrin mRNA, and this motif is necessary for its localization to adhesion complexes at the periphery of human cells in culture (Adereth et al. 2005). Whether this motif functions as a zipcode out of context of either the β -actin or α_3 integrin transcripts is not known.

Like the aforementioned examples, some transported mRNAs contain multiple redundant zipcodes. However, there are at least two examples of systems where multimerization of single elements or combinations of distinct elements are not required to direct localization. In *Xenopus*, the *fatvg* zipcode (FVLE1) displays efficient localization when present in a single copy (Chan et al. 1999). In addition, one bud-localized RNA in yeast, *ASH1*, contains four zipcodes (three in the coding region and one in the 3' UTR), each of which is sufficient for localizing a reporter RNA when fused to the 3' end (Chartrand et al. 1999; Gonzalez et al. 1999). Similarly, each of the two zipcodes from bud-localized *WSC2* localizes a reporter construct with full efficiency (Jambhekar et al. 2005). In context of the native *ASH1* RNA, however, all four zipcodes are necessary for full levels of localization (Gonzalez et al. 1999). Because the reporter mRNAs used in these studies were likely not translated, it is not clear whether the requirement for multiple zipcodes reflects long-range interactions with surrounding sequences or whether passage

of ribosomes along the mRNA impairs zipcode activity (see below).

Although repeats of minimal motifs (e.g., VM1, BLE1, +2' ME) can often substitute for intact zipcodes in localizing RNAs, it remains unclear exactly how these repeated sequences compensate for the loss of other essential zipcode elements. For example, how does multimerization of a fragment from stem-loop V of the *bicoid* 3' UTR (BLE1) compensate for the loss of stem-loop IV? One possibility is that the various RNA-binding proteins needed for localizing a particular transcript can bind weakly to each other as well as to RNA. Creating a high-density cluster of one protein via multimerization of its binding site may recruit other essential proteins to the mRNP via protein-protein, rather than protein-RNA, interactions. In support of this possibility, clusters of VM1 and E2 sites in Vg1 RNA recruit 40LoVe, a component of the localizing mRNP (Czaplinski and Mattaj 2006). Arn et al. (2003) have proposed that specific RNA recognition involves multiple low-affinity and low-specificity interactions that can occur either on a complex RNA target or on tandem repeats of a minimal recognition element.

ROLE OF RNA PROCESSING IN ZIPCODE FUNCTION

It is generally believed that mRNA processing is not required for zipcode recognition by transport complexes, although some studies have indicated that the two may not be completely independent. Experiments involving injection of zipcode RNAs into oocytes suggest that processing is not required for zipcode recognition, as these RNAs localize efficiently in the absence of 5' capping, poly(A) tail addition, or splicing (e.g., Glotzer et al. 1997). The vegetally localized *Xenopus Xlsirt* RNA is noncoding (Kloc et al. 1993) and likely not subject to mRNA processing. In yeast, expressing zipcode RNAs under the Pol III promoter (which eliminates standard mRNA processing events) also does not interfere with recognition by the transport complex (Long et al. 2000; Jambhekar et al. 2005; Olivier et al. 2005) or localization (Beach and Bloom 2001). Although localization of cyclin B mRNA to pole cells and somatic nuclei during *Drosophila* embryogenesis (Whitfield et al. 1989; Raff et al. 1990) is influenced by splicing, there does not appear to be a mechanistic relationship between the two processes: the unlocalized spliced isoform simply lacks the 3' UTR zipcode present in the localized transcript (Dalby and Glover 1992).

A detailed analysis of *osk* localization, however, revealed a mechanistic role for splicing in directing transport. *osk* transgenes, expressed in a background in which no endogenous *osk* is produced, required splicing at the first of three exons for localization to the posterior during late stages of oogenesis (Hachet and Ephrussi 2004). Localization was independent of intron sequence, as replacement of the first

intron sequence with that of the third intron supported localization. Although exon–junction complexes are presumed to be assembled at each of the three introns in *osk*, assembly is required only at the first junction for localization. Interestingly, only splicing at the first intron supports colocalization of *osk* RNA with Y14 (Hachet and Ephrussi 2004), a component of the exon–junction complex and an essential factor in *osk* transport (Hachet and Ephrussi 2001). Surprisingly, the requirement for splicing seems to function in *trans*: a LacZ reporter fused to the 3' UTR of *osk* (which contains zipcode regions) (Kim-Ha et al. 1993) localizes efficiently only when endogenous *osk* is expressed (Hachet and Ephrussi 2004). In support of the role of splicing in mediating localization, components of exon–junction complexes (e.g., Y14 and mago) (Hachet and Ephrussi 2001; Le Hir et al. 2001; Mohr et al. 2001), as well as factors involved in nonsense-mediated decay (e.g., eIF4AIII) (Palacios et al. 2004), are required for *osk* localization. It is not clear, however, whether these proteins play dual roles in splicing or transport, or whether splicing is simply a prerequisite for nuclear export and therefore cytoplasmic localization. In a separate study, insertion of a zipcode (the TLS region from the anteriorly localized *Drosophila K10* transcript) near a 5' splice site supported localization but impaired splicing (Cohen et al. 2005). Whether this result reflects simple steric interference between the localization and splicing machineries or a more complex mechanistic relationship remains unknown. It is likely that other localized transcripts also require mRNA processing factors for transport.

In addition to the connection between splicing and localization, there is evidence that mRNAs are “marked” for transport prior to nuclear export. hnRNP I, a component of the *Vg1* and *VegT* transport mRNPs, associates with both mRNAs in the nucleus and remains bound to them in the cytoplasm. Prrp and Xstau, other essential components of the transport complex, are recruited to the RNAs in the cytoplasm, presumably via their interactions with hnRNP I (Kress et al. 2004). Thus, binding of shuttling factors to mRNA in the nucleus can guide the assembly of a transport complex in the cytoplasm. In yeast, She2 may perform a function similar to hnRNP I. She2 shuttles between the nucleus and cytoplasm, and its nuclear export requires binding to RNA (Kruse et al. 2002), suggesting that transported RNAs emerge from the nucleus with She2 already bound. She2 could then recruit She3 and Myo4 to effect transport (Kruse et al. 2002). In addition to the core–She complex components, Loc1, a nuclear protein that binds double-stranded RNA nonspecifically, is also essential for bud localization of *ASH1* (Long et al. 2001). Loc1 was recently shown to be important for rRNA processing as well as nuclear export of 60S ribosomal subunits, and it was hypothesized that aberrant nuclear–cytoplasmic shuttling or defects in translation may impair *ASH1* localization (Urbinati et al. 2006).

Cytoplasmic mRNA processing may also affect zipcode activity. In yeast, anchoring of *ASH1* to the bud tip requires translation (Gonzalez et al. 1999). Surprisingly, the translation and localization processes seem to act antagonistically. All four *ASH1* zipcodes are necessary for full levels of localization of the native RNA (Gonzalez et al. 1999) or translatable forms of the RNA with zipcodes ectopically located in the UTR (Chartrand et al. 2002). However, only one zipcode is sufficient for localizing a nontranslatable reporter (Beach and Bloom 2001; Jambhekar et al. 2005), suggesting that translation impairs zipcode activity. In support of this finding, deletion of associated translation inhibitors Khd1 or Puf6 impairs localization (Irie et al. 2002; Gu et al. 2004). Because yeast zipcodes generally lie in coding regions, it is possible that ribosomes compete with the She complex for zipcode occupancy; the presence of four zipcodes in *ASH1* may ensure that at least one remains bound to the She complex at any time. The secondary structure of the zipcodes delays passage of ribosomes along the RNA (Chartrand et al. 2002), thus ensuring that the RNA is not translated prior to its localization in the bud. A similar translation-based mechanism may inhibit dendritic localization of RNAs in neural cells: transport to dendrites appears to be the “default” state, and association with ribosomes restricts RNAs to the cell body (Lu et al. 1998). Analogously, a 1 kb region in the MBP 3' UTR is required (in addition to the A2RE-containing zipcode) to localize a protein-coding reporter RNA to the myelin compartment of oligodendrocytes. However, the A2RE element is sufficient for localizing a non-protein-coding construct (Ainger et al. 1997). In these cases, it is not clear how zipcodes (which are generally located in the 3' UTR) can overcome translation-dependent inhibition of transport.

Translation also influences the localization of vasopressin RNA, which is localized to both dendrites and axons of neurons. Two fragments, one entirely in the coding region and the other overlapping the ORF and 3' UTR, act synergistically to localize the RNA to dendrites (Prakash et al. 1997). Alleles of the gene containing a single base (guanosine) deletion in the downstream zipcode impair axonal but not dendritic localization (Mohr et al. 1995). Zipcodes mediating axonal localization of this RNA have not been identified. It is possible that the downstream dendritic targeting signal also mediates axonal localization, and that the guanosine deletion impairs recognition specifically by the axonal transport complex. The authors, however, proposed that the loss of a stop codon caused by the frameshift mutation impaired release of the transcript from ribosomes, thus preventing recognition by the transport machinery (Mohr et al. 1995). Translational regulation is critical for localization in several other systems, although the mechanisms likely involve *trans*-acting factors rather than direct interaction of ribosomes and RNA zipcodes (see, e.g., Wilhelm et al. 2003; Yano et al. 2004).

Factors promoting RNA stability may also play a role in localization. In the case of tau mRNA, a U-rich region in the 3' UTR that binds HuD and increases RNA stability (Aranda-Abreu et al. 1999) is also required for axonal localization (Aronov et al. 2001). However, it is not clear whether HuD is an essential component of the transport complex, or whether, in the absence of HuD, the RNA is simply degraded before it can be transported. At this time it is not known whether transported RNAs are generally more or less stable than their nontransported counterparts, or even whether they are degraded by similar mechanisms.

Regulation of zipcode activity

With a few notable exceptions (see below), zipcodes generally function constitutively, providing the cytoskeletal structure is intact and appropriate *trans*-acting factors are present. In yeast, disruption of actin filaments causes symmetric distribution of bud-localized RNAs (Jansen et al. 1996; Long et al. 1997; Takizawa et al. 1997, 2000), and dissolution of actin cables late in mitosis causes bud-tip localized RNAs to migrate to the bud neck (Beach et al. 1999). β -actin RNA localization to fibroblasts also requires actin microfilaments (Sundell and Singer 1991), while RNA transport in *Drosophila* (Pokrywka and Stephenson 1995) and *Xenopus* (Yisraeli et al. 1990) oocytes and β -actin RNA localization in nerve growth cones (Bassell et al. 1998) require microtubules. Actin filaments have also been implicated in anchoring localized RNAs in yeast, *Xenopus*, and fibroblasts (Yisraeli et al. 1990; Sundell and Singer 1991; Beach and Bloom 2001; Liu et al. 2002). Therefore, cytoskeletal rearrangements during cellular development can alter the distribution of RNAs, although cytoskeletal regulation may not be sufficient for proper RNA targeting in all cases (Theurkauf and Hazelrigg 1998).

Surprisingly, some zipcodes can function in cellular contexts different from those in which they are normally active, indicating that there is minimal negative regulation of zipcode function. This phenomenon has been demonstrated most extensively in *Drosophila*. RNAs that are asymmetrically localized in oocytes accumulate at the apical region when injected into embryos, in a pattern similar to pair-rule transcripts (Bullock and Ish-Horowitz 2001). These transcripts localize identically in embryos even though they exhibit different localization programs in oocytes. Furthermore, *cis*-acting mutations that impair localization in oocytes also impair apical transport in embryos (Bullock and Ish-Horowitz 2001; Snee et al. 2005), suggesting that the localization machinery in both settings recognizes similar zipcodes. However, proteins known to be part of the ovarian *bicoid* transport complex (e.g., Swallow, Modulo, poly[A]-binding protein, Smooth, Nod) (Arn et al. 2003) were not detected in a fractionated *bicoid*-binding embryonic extract (Snee et al. 2005). It has been proposed that zipcode recognition by the transport complex results

in each case from multiple low-affinity and low-specificity interactions (Arn et al. 2003; Snee et al. 2005). However, there do not generally appear to be combinatorial control mechanisms for repressing zipcode activity as there are for repressing DNA promoter elements (Istrail and Davidson 2005).

One exception is the localization of β -actin mRNA, which is subject to positive and negative control mechanisms. In fibroblasts, this mRNA is localized to the leading edge by zipcode(s) located in the 3' UTR (see above). Localization was stimulated by PDGF treatment or by serum addition following starvation. Both chemical inhibition and activation of PKA or PKC, as well as decreasing cellular ATP levels, reduced localization (Latham et al. 1994). The investigators proposed that the cellular phosphorylation status regulates β -actin mRNA localization. Similarly, β -actin mRNA localization to nerve growth cones is regulated by extracellular signals. Addition of neurotrophin-3 (NT-3) stimulated localization, as did activation of adenylate cyclase (Zhang et al. 2001). In both systems, it is not clear how signaling pathways regulate β -actin mRNA transport. The simplest explanation is that *trans*-acting factors required for transport are regulated by phosphorylation. Given the rapid (within 2 min) localization of β -actin mRNA in response to the appropriate stimuli (Latham et al. 1994) and the fact that β -actin mRNA is transported to leading edges in the presence of translation inhibitors (Sundell and Singer 1990), it is unlikely that *de novo* synthesis of mRNP components is required for transport. It is possible, however, that repressors of transport are bound to negatively acting RNA elements in situations where the RNA is not localized, and that extracellular stimuli promote zipcode function by removing these repressors. Identifying the entire complement of β -actin mRNA-bound proteins under localizing and nonlocalizing conditions in the two cell types will help to elucidate how localization of this transcript is regulated.

The localization of α CaMKII also appears to be under positive and negative regulation, and *cis*-acting elements responsible for this regulation have been defined. The zipcode for this transcript was mapped to the 3' UTR (Mayford et al. 1996), and subsequent fine-mapping studies yielded three positive *cis*-acting regions. Blichenberg et al. (2001) identified an \sim 1200 nt fragment from the latter half of the 3' UTR as being sufficient for localizing an EGFP reporter. Another zipcode was mapped to the distal 170 nt of the UTR, which contains cytoplasmic polyadenylation elements (CPEs) (Huang et al. 2003). Zipcode activity of this region required WT cytoplasmic polyadenylation element-binding protein (CPEB). Because many transcripts (both localized and nonlocalized) contain CPEs, the investigators proposed that the structural context of the CPE may determine whether it functions as a zipcode. Furthermore, because the CPE mediated localization when placed

in a polylinker sequence, the investigators concluded that the CPE may be a default transport signal that is masked by additional negative-regulatory *cis*-acting signals in nonlocalized transcripts (Huang et al. 2003). A third zipcode was reported within the first 94 nt of the 3' UTR. A 30 nt sequence within this region showed high homology with the 3' UTR of neurogranin (another dendritically localized RNA), and this motif was necessary for dendritic localization of 3' UTR sequences derived from both α CaMKII and neurogranin (Mori et al. 2000).

Two potential repressors of the 5' 94 nt zipcode have been reported. One repressor was proposed to lie between nucleotides 94 and 725 (as the first 725 nt did not show zipcode activity), and an enhancer was postulated to lie between nucleotides 725 and 831 (as nucleotides 1–831 did localize to dendrites) (Blichenberg et al. 2001). A simpler explanation for these contradictory findings may be that truncating the 3' UTR at 725 nt causes the 94 nt zipcode to adopt a conformation that is refractory to recognition by the transport complex. Another repressor was proposed to lie between nucleotides 831 and 1497 of the 3' UTR, based on the finding that the first 831 nt localized to dendrites, while the first 1497 did not. Depolarizing neurons by addition of KCl relieved inhibition and restored dendritic localization of nucleotides 1–1497 of the 3' UTR (Mori et al. 2000). This finding was unexpected because localization of full-length α CaMKII (unlike that of BDNF and TrkB, whose dendritic localization is sensitive to depolarization) (Tongiorgi et al. 1997) is constitutive (Burgin et al. 1990). In the native RNA, the zipcodes identified by Blichenberg et al. (2001) and Huang et al. (2003) may bypass the requirement for depolarization. A Y-element in the protein-coding region of the transcript, which is essential for transport and binds TB-RBP (a protein implicated in both localization and translation inhibition) (Severt et al. 1999), may also relieve repression. These positive elements were not present in the KCl-sensitive 1–1497 construct. It is not clear whether depolarization causes a conformational change in the RNA itself, a change in the expression/activity of any *trans*-acting factors, or both. Analogous to the putative transport repressors in α CaMKII, the VM1 sites in the *Vg1* zipcode have been proposed to repress transport, since injection of VM1-containing repeat RNA (which titrates out hnRNP I) enhanced *Vg1* localization (Czaplinski and Mattaj 2006). Conclusive demonstration of *cis*-acting inhibitors of transport awaits isolation of sequences that can prevent localization out of context of their native RNAs.

Sequence and structural basis of RNA recognition

Despite the large number of zipcodes isolated in a variety of systems, no clear patterns in zipcode sequence or structure have emerged. Some zipcodes appear to be recognized by multiple transport complexes. For example, a 300 nt

zipcode from the vegetally localized *Xenopus XNIF* mRNA (Claussen et al. 2004), as well as a 137 nt zipcode from *Xlsirt* (Allen et al. 2003), are recognized by proteins that participate in both the METRO and late transport pathways. The *K10* zipcode, as well as three other transcripts that are transported in *Drosophila* oocytes, localize apically when injected in blastoderm embryos (Bullock and Ish-Horowicz 2001). Conversely, some transport complexes recognize multiple zipcodes. *Xcat-2* and *Xlsirt*, for example, are both localized by the METRO pathway (see above), but the 137 nt zipcode in *Xlsirt* is not found in *Xcat-2* (Allen et al. 2003). Few zipcodes have been minimized sufficiently to allow detailed analysis of the exact requirements for activity. The dendritic targeting element in MAP2 mRNA, which is localized to dendrites of neurons, comprises a 640 nt region in the 3' UTR, which is predicted to fold into multiple stem-loops (Blichenberg et al. 1999). However, it is not known which sequence/structural subelements are necessary for localization. Furthermore, the RNA-binding proteins that recognize zipcodes remain unknown in many cases, making analysis of the physical RNA–protein interactions impossible.

An attractive model for zipcode recognition is that *trans*-acting factors recognize a specific secondary structure in the RNA, often a hairpin stem-loop structure, along with a small number of specific nucleotides (Chartrand et al. 1999). This model has largely proven to be correct in the case of the TLS element (Cohen et al. 2005), a zipcode present in both the *orb* and *K10* transcripts of *Drosophila* (Serano and Cohen 1995). The native zipcode is predicted to fold in a stem-loop consisting of a 17 base pair (bp) stem interrupted by two single-base bulges and an 8 base loop (Cohen et al. 2005). Fine-mapping studies showed that increasing loop size or decreasing the length of the stem interfered with transport/localization. Interestingly, some compensatory mutations in the stem supported localization while others did not. Mutations that altered the stereochemistry of the minor groove (e.g., U·A \rightarrow C·G or G·C) impaired localization, but those that preserved minor groove stereochemistry (e.g., U·A \rightarrow A·U) had no effect. The only exception was at the fifth position of the stem, where the U·A \rightarrow A·U mutation caused subtle defects in localization. It appears, therefore, that the TLS element is recognized on the basis of hydrogen-bond patterns in the minor groove of the helix, with a small contribution from the major groove of base-pair 5 (Cohen et al. 2005).

Like the TLS element, the dendritic targeting signal at the 5' end of the BC1 RNA (Muslimov et al. 1997) also folds into a single stem-loop (Rozhdetsvensky et al. 2001). This finding was surprising because, based on sequence similarity to tRNA, BC1 was expected to adopt a cloverleaf structure. Although tRNAs are also localized to dendrites, some of them remain to function in the cell body. It was hypothesized that the difference in structure allows BC1 to be transported more

efficiently into dendrites than are tRNAs (Rozhdestvensky et al. 2001). The specific sequence and structural properties of BC1 that allow its transport remain undefined. Therefore, it is not clear whether any hairpin bearing structural similarity to BC1 is competent for localization.

A 75 nt zipcode from the 3' UTR of *Xvelo1*, a transcript vegetally localized by the late pathway in *Xenopus* oocytes, is also predicted to fold into a hairpin structure (Claussen and Pieler 2004). Detailed analysis of this zipcode, however, revealed an RNA-recognition strategy more complicated than simple hairpin binding. Deletion of a largely single-stranded 5' tail abolished localization, indicating that the hairpin alone is insufficient for localization. Second, compensatory mutations in the stem region (which maintain the predicted secondary structure of the RNA) did not support WT levels of localization. Because the compensatory mutations maintained the architecture of the minor groove of the helix, it appears that the *Xvelo1* zipcode is not recognized by a TLS-like mechanism. It is possible that nucleotide identities in the stem are important for recognition of *Xvelo1* by the transport machinery, or that these bases contribute to forming a specific three-dimensional structure necessary for recognition.

Two transcripts that are localized to the dorsoanterior corner of *Drosophila* oocytes, *gk* and I factor, share similar hairpin zipcodes that function during stage 8 of oogenesis (see above) (Van De Bor et al. 2005). While both zipcodes contain three stem regions separated by two internal bulged loops, the precise stem lengths, bulge sizes and locations, and terminal loop lengths differ, as do the nucleotide identities. Because detailed mutagenesis studies have not been performed on these zipcodes, the molecular mechanism by which any shared *trans*-acting factors could recognize such a diversity of substrates, yet maintain specificity for their targets, remains unclear.

Zipcodes mediating bud localization of mRNAs in yeast have, perhaps, been most well characterized (Chartrand et al. 1999; Gonzalez et al. 1999; Jambhekar et al. 2005; Olivier et al. 2005). These sequences, like the stem-loop IV–V zipcode in the *Drosophila bicoid* RNA (Macdonald and Kerr 1998), reveal a primary sequence as well as structural requirements. The isolation of multiple short zipcodes recognized by a single transport complex (She2/3) (Jambhekar et al. 2005) has provided insight into the rules as well as the exceptions governing She2/3 recognition of target RNAs. A core single-stranded CG dinucleotide (Jambhekar et al. 2005), often present as a CGA triplet (Olivier et al. 2005), appears essential for transport, while a stretch of downstream adenosines contributes to, but is not essential for, She2/3 recognition (Jambhekar et al. 2005). Although the primary sequences of the stem regions of different zipcodes vary, these sequences contribute to recognition in some (but not all) cases (Jambhekar et al. 2005). In some cases, compensatory mutations in stem regions maintain activity (Chartrand et al. 1999; Gonzalez

et al. 1999; Jambhekar et al. 2005; Olivier et al. 2005), whereas in other cases they do not (Jambhekar et al. 2005). A nonessential bulged cytosine also enhances zipcode recognition when present (Jambhekar et al. 2005; Olivier et al. 2005), and this enhancement depends on the distance between the cytosine and CG dinucleotide (Olivier et al. 2005). The variable contributions of different sequence and secondary structural features suggest that the She complex recognizes hairpin zipcodes on the basis of tertiary structures as well as primary sequence and secondary structure. Although the hairpin mechanism initially proposed for recognition of She2-dependent zipcodes was attractive, subsequent analyses reveal a more complex recognition strategy. Because many nonlocalized transcripts are also predicted to fold into multiple hairpin structures (A. Jambhekar and J.L. DeRisi, unpubl.), the secondary-structure-recognition model is not sufficient to explain the specific recognition of zipcodes by She2/3. Furthermore, it has not been possible to engineer novel zipcodes by incorporating the essential primary sequence and secondary structural motifs elucidated by comparative and mutational analyses (A. Jambhekar and J.L. DeRisi, unpubl.), indicating that these requirements alone do not define a She2-dependent zipcode. Although the crystal structure of She2 revealed residues important for RNA binding and dimerization (Niessing et al. 2004), it is not clear how the She complex might recognize its target zipcodes with high affinity and specificity. A three-dimensional structure of the RNA–protein complex will be necessary to elucidate the roles of various RNA sequence and structural features in binding.

Although analysis of most zipcodes has focused on sequence and secondary structural elements, the higher-order structure of the *bicoid* 3' UTR is essential for its embryonic localization, suggesting that complex structures also may be important for other zipcodes. An intermolecular dimerization event generates a quaternary structure that directs recognition and localization to astral microtubules during early stages of embryogenesis by Staufen (Ferrandon et al. 1997). Dimerization of the UTR initiates via base pairing between single-stranded loops in domain III, and is then stabilized by surrounding sequences (Wagner et al. 2001). Staufen contains five double-stranded RNA-binding domains (dsRBDs), of which three bind RNA and two do not (Micklem et al. 2000). The NMR structure of dsRBD3 complexed with RNA reveals binding of the domain to an RNA monomer (Ramos et al. 2000); it is not clear whether the *bcd* 3' UTR dimer is recognized by one or more intact molecules of Staufen. RNA dimerization may serve to nucleate higher-order RNA–protein “packages” that function as the substrates for efficient mRNP transport. The characterization of a mechanism for RNA dimerization provides a good starting point for examining the formation and structures of transported RNA packages.

Predicting zipcodes

Because of the complexity of and variations in RNA–protein recognition strategies employed by different transport systems, faithful *in silico* predictions of zipcode regions have proven to be quite difficult. Furthermore, the lack of sufficient numbers of zipcodes recognized by a given transport system, combined with the difficulties of predicting higher-order RNA structures, have impeded progress in this area.

Predicting zipcodes that can be defined only by primary sequence has been the most successful. For example, a dendritic targeting sequence in neurogranin RNA was identified based on sequence similarity to that of α -CaMKII mRNA (Mori et al. 2000). In *Xenopus*, clusters of CAC motifs have been identified as zipcode regions (Betley et al. 2002). The CAC motif was first identified in the context of an E2 element, which constitutes a Vera binding site (Deshler et al. 1998), and later in the *VegT* RNA, which also localizes in a Vera-dependent manner (Kwon et al. 2002). A search for repeated motifs in the 3' UTRs of nine other vegetally localized RNAs generated short motifs containing CAC triplets, with statistically significant enrichments of the motifs in zipcode regions when compared with the surrounding sequences (Betley et al. 2002). CAC-containing regions from *Xpat* and *Xcat-2* 3' UTRs, as well as from vegetally localized RNAs in ascidian embryos, were necessary and sufficient for localizing reporter constructs in *Xenopus* eggs. Surprisingly, the CAC zipcodes were found in transcripts localized by either the METRO or late pathways, suggesting that the motif was not pathway specific but may be recognized by a protein that functions in both pathways. Although Vera binds to zipcodes from transcripts localized by both the early and late pathways (Choo et al. 2005), the binding is dependent on CAC motifs in only some cases (Betley et al. 2002). The exact mechanism of CAC zipcode recognition in the early and late pathways remains unclear.

Identifying zipcodes that are recognized on the basis of structure as well as sequence has proven more difficult. One powerful method of identifying functional RNA elements is to search for evolutionarily conserved RNA secondary structures in related species, since essential secondary structures are predicted to be conserved while primary sequences will vary. Thus, the presence of compensatory mutations in related RNAs will signify structures under selective pressure. Algorithms such as FOLDALIGN (Gorodkin et al. 1997) and X2s (Juan and Wilson 1999), as well as manual inspection of sequences, have been used to identify functional elements in HuR target mRNAs and noncoding telomerase RNAs (Romero and Blackburn 1991; Dandjinou et al. 2004; Lopez de Silanes et al. 2004). Such analyses have been less successful in identifying zipcode regions. Many algorithms, such as CONSTRUCT (Luck et al. 1999), require good sequence alignments for accurate

structure prediction, making it difficult to identify short zipcode elements buried in long UTRs or coding regions. Manual comparison of the predicted secondary structures of *bicoid* 3' UTRs from six different species of *Drosophila* yielded globally similar structures (Macdonald 1990). The details of stem and loop lengths and sequences, however, varied between species, making it difficult to pinpoint putative transport complex binding sites (Macdonald 1990). At the same time, the low sequence divergence between the species precluded using a phylogenetic approach due to insufficient numbers of compensatory mutations (Ferrandon et al. 1997). Comparison of the *nanos* 3' UTRs from *D. melanogaster* and *D. virilis* revealed several conserved sequence elements both within and outside the four zipcode regions, as well as a translational control element (TCE) that showed both sequence and structural conservation (Gavis et al. 1996a,b; Crusis et al. 2000). Mapping of zipcodes in both *bicoid* and *nanos* was initially achieved by testing various 3' UTR fragments for localization, presumably because the sequence and structural similarities between the available sequences of homologous genes were not sufficient to identify candidate zipcode regions (Macdonald 1990; Gavis et al. 1996a).

In addition to comparing RNA sequences and structures between related species, analysis of multiple targets of a transport complex from a single species also has the potential to elucidate essential zipcode features. In *Xenopus*, both the *VegT* and *Vg1* RNAs are vegetally localized by the late pathway. The 3' UTRs of both transcripts reveal clusters of E2 and VM1 sites (Bubunenko et al. 2002) that bind Vera and hnRNP I, respectively. These clusters are similar in length and are located at similar positions in the 3' UTRs, although the exact numbers and locations of each type of site vary between the two UTRs. It was proposed that clusters of E2 and VM1 sites define a vegetal localization element (Bubunenko et al. 2002). A similar analysis was attempted in yeast, using four She complex target zipcodes from the *ASH1* gene (Olivier et al. 2005). After deriving rules for She-complex recognition, a search for novel zipcodes in 23 other known bud-localized RNAs yielded four additional putative zipcodes, of which two localized *in vivo*. Given that bona fide zipcodes fitting the search criteria, such as the one validated from *WSC2* (Jambhekar et al. 2005), were missed, it is clear that the available tools for RNA structure prediction are not sufficiently accurate to reliably identify zipcodes within large data sets of sequences. This finding is not surprising, given that analysis of additional She-complex-dependent zipcodes revealed that variations in sequence/structure can be tolerated in some contexts (Jambhekar et al. 2005), which indicates that the initial rules derived for She-complex recognition (Olivier et al. 2005) do not accurately describe the range of zipcodes that can be recognized by this system.

Despite the recent advancements in techniques for visualizing RNA distribution, zipcode identification and

characterization remain a tedious task. Elucidating essential zipcode features remains problematic because it is often impossible to test sequence and structural requirements independently. Although large zipcode-containing regions function out of context, the detailed requirements for activity often appear context dependent; thus, it is likely that the majority of zipcodes are recognized on the basis of tertiary structural features. Because motif-finding algorithms have not proven to be efficient at identifying zipcodes, the only genome-wide method for identifying RNA targets is RNA-IP, which requires prior knowledge of the RNA-binding protein (Takizawa et al. 2000). To date, there is no available three-dimensional structure of a transport complex component bound to its native RNA target. Better knowledge of the precise sequences and higher-order structures necessary for zipcode activity in various systems, as well as the development of algorithms that predict such structures, will aid in identifying novel localized transcripts and in elucidating the spatial and temporal regulation of RNAs from the time of their synthesis in the nucleus to their degradation in the cytoplasm.

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