A small predicted stem-loop structure mediates oocyte localization of Drosophila K10 mRNA

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SUMMARY

The establishment of dorsoventral polarity in the Drosophila oocyte and future embryo is dependent on the efficient transport of K10 mRNA from nurse cells into the oocyte. To investigate the cis-requirements of K10 mRNA transport, we used a transgenic fly assay to analyze the expression patterns of a series of K10 deletion variants. Such studies identify a 44 nucleotide sequence within the K10 3′ untranslated region that is required and sufficient for K10 mRNA transport and subsequent localization to the oocyte’s anterior cortex. An inspection of the 44 nucleotide transport/localization sequence (TLS) reveals a strong potential for the formation of a stem-loop secondary structure. Nucleotide substitutions that interfere with the predicted base-pairing do not block mRNA transport or anterior localization. Conversely, mutations that alter the base composition of the TLS while maintaining predicted base-pairing do not block mRNA transport or anterior localization. We conclude that K10 mRNA transport and anterior localization is mediated by a 44 nucleotide stem-loop structure. A similar putative stem-loop structure is found in the 3′ untranslated region of the Drosophila orb mRNA, suggesting that the same factors mediate the transport and anterior localization of both K10 and orb mRNAs. Apart from orb, the K10 TLS is not found in any other localized mRNA, raising the possibility that the transport and localization of other mRNAs, e.g., bicoid, oskar and gurken, are mediated by novel sets of cis- and trans-acting factors. Moreover, we find that the K10 TLS overrides the activity of oskar cis-regulatory elements that mediate the late stage movement of the mRNA to the posterior pole. We propose the existence of a family of cis-regulatory elements that mediate mRNA transport into the oocyte, only some of which are compatible with the elements that mediate late stage movements.

Key words: Drosophila K10 gene, mRNA localization, mRNA transport, Drosophila oogenesis, RNA secondary structure

INTRODUCTION

Examples of mRNA localization as a means for protein targeting have increased greatly in recent years (reviewed in Macdonald, 1992; Ding and Lipshitz, 1993; St Johnston, 1995). It is a particularly common mechanism in developmental systems, where it is often desirable to store positional information in an inactive form. It is also widely used in large cells, e.g., eggs and neurons, possibly because it is more energy efficient to localize a relatively small number of mRNAs and translate them many times than it is to independently localize a large number of protein molecules. Despite its widespread occurrence and biological importance, the mechanism of mRNA localization is poorly understood compared to our understanding of other protein targeting mechanisms (e.g., see Pelham and Munro, 1993).

The richest identified source of localized mRNA is the Drosophila oocyte, in which over a dozen localized mRNAs have been found (reviewed in Macdonald, 1992; Ding and Lipshitz, 1993). The localization of these mRNAs is a dynamic process that begins with mRNA synthesis in nurse cells and transport to the oocyte’s posterior pole. mRNA synthesis and transport are tightly coupled events inasmuch as mRNAs do not accumulate in nurse cells to detectable amounts. In most cases, mRNA synthesis and transport extend from just prior to egg chamber formation though ~stage 7 of oogenesis (see King, 1970, for a complete description of egg chamber formation and the ensuing 14 stages of egg chamber maturation). At stage 8, transported mRNAs are moved from the oocyte’s posterior pole to the oocyte’s anterior cortex, where mRNA-specific sorting events occur. For example, oskar (osk) and gurken (grk) mRNAs are moved to the oocyte’s posterior pole and anterodorsal corner, respectively, during late stage 8/early stage 9 (Kim-Ha et al., 1991; Ephrussi et al., 1991; Neuman-Silberberg and Schüpbach, 1993). Other mRNAs, e.g., fs(1)K10 (K10), bicoid (bcd) and orb, remain at the oocyte’s anterior cortex until stage 10B or later (Cheung et al., 1992; Berleth et al., 1988; Lantz et al., 1992).
Pharmacological studies indicate that mRNA transport and subsequent localization events within the oocyte are mediated by microtubules (Pokrywka and Stephenson, 1991, 1995; Theurkauf et al., 1993). Each nurse cell–oocyte complex is connected by a single microtubule array (Theurkauf et al., 1992). Through stage 6, the array’s organizing center (minus-end) is positioned at the oocyte’s posterior pole, and its plus-ends extend into nurse cells. During stages 6-8, this array is dismantled and a new one, nucleated along the oocyte’s anterior cortex and terminated at the oocyte’s posterior pole, is formed (Theurkauf et al., 1992). The dynamics and polarity of microtubules suggest that mRNA transport and anterior localization are both powered by a minus end-directed motor, e.g., a member of the dynein superfamily, while late stage (i.e., stage 9) movement of certain mRNAs to the oocyte’s posterior pole is powered by a plus-end, kinesin-like, motor. Consistent with this idea, kinesin’s motor subunit, when fused to β-galactosidase (β-gal), targets β-gal enzyme activity to the posterior pole of stage 9 and older oocytes (Clark et al., 1994).

The observed overlap between the localization patterns of different Drosophila mRNAs is suggestive of a modular design of cis-regulatory elements. Specifically, one might expect all localized mRNAs to contain an element that mediates association with a minus-end motor. Some mRNAs, like osk, that move to the oocyte’s posterior pole at stage 9, might be expected to also contain an element that mediates association with a plus-end motor. Additional elements that mediate the association of mRNAs to non-microtubule components of the cytoskeleton might be required to maintain localization of some mRNAs, particularly those that remain localized during cytoplasmic streaming stages (i.e., stages 11-12), when microtubules are reorganized yet again (Theurkauf et al., 1992). In direct support of a modular design of mRNA localization elements, Kim-Ha et al. (1993) have identified distinct elements within the osk 3' untranslated region (3'UTR) for mRNA transport and for movement to the oocyte’s posterior pole. Distinct regulatory elements have also been identified in the bcd 3'UTR; the 53 nucleotide BLE1 mediates bcd mRNA transport and localization to the oocyte’s anterior cortex, while three non-contiguous sequences mediate late-stage stage-dependent localization to the oocyte’s anterior cytoplasm (Macdonald et al., 1993; Ferrandon et al., 1994).

In the most streamlined version of a modular regulatory logic for mRNA localization, all similar localization steps would be mediated by the same set of cis- and trans-regulatory factors. However, this is apparently not the case. For example, bcd mRNA transport, but not the transport of other examined mRNAs, is mediated by exuperantia gene activity (Berleth et al., 1988; St Johnston et al., 1989). Furthermore, there are no obvious sequence homologies between the localization control regions of similarly localized mRNAs. The lack of such sequence homology could, however, simply reflect the physical nature of mRNA localization elements. For example, they may specify RNA secondary structures, which could be difficult to detect by conventional searching algorithms – both because many different sequence combinations can specify a particular structure and because a single structure could be composed of widely separated sequences. Thus, the degree to which the similar movements of different mRNAs are controlled by the same elements remains unknown. In cases where different mRNAs do use different elements for similar movements, it is of interest to know if the different elements have evolved for trivial or functionally significant reasons.

In this paper, we identify and characterize the cis-regulatory sequences of K10 mRNA localization. K10 mRNA is synthesized in nurse cells, transported to the oocyte’s posterior pole and moved to the oocyte’s anterior cortex at stage 8, where it persists through stage 10B (Cheung et al., 1992). Consistent with K10’s relatively simple mRNA localization pattern, we find that a single 44 nucleotide sequence located within the K10 3'UTR is necessary and sufficient for all K10 mRNA localization steps. The activity of this sequence, called the transport/localization sequence (TLS), is apparently dependent on its folding into a stem-loop secondary structure. All mutations that disrupt TLS’s predicted base-pairing pattern also disrupt its mRNA transport and anterior localization activity. Conversely, all mutations that alter TLS’s primary sequence, while maintaining its predicted base-pairing pattern, have little or no adverse affect on mRNA transport and anterior localization. The small size of the TLS, coupled with the observation that all mutations that decrease the TLS’s transport activity similarly decrease its anterior localization efficiency, suggests that the TLS is a single regulatory element. That is, the TLS appears to interact with a single regulatory protein or protein complex, presumably one that facilitates association of the mRNA with a minus end-directed microtubule-based motor protein (see above). A sequence structurally similar to TLS is also found in orb mRNA, which exhibits an mRNA distribution pattern similar to that of K10 (Lantz et al., 1992), suggesting that TLS is a conserved element mediating mRNA association with a minus-end motor. Curiously, the TLS apparently overrides the posterior localization control sequences of osk mRNA: the substitution of the TLS for osk’s putative transport control sequences renders osk’s posterior localization control sequences inactive. We suggest that mRNAs that have different final destinations within the oocyte require functionally different transport control elements.

**MATERIALS AND METHODS**

**Fly stocks**

The wild-type stock is Oregon R. The K10 allele is K10<sup>F<sub>4</sub>mo</sup>, which was provided by T. Schüpbach and is described in Cheung et al. (1992). All markers and balancers are described in Lindsley and Zimm (1992).

**Transformation constructs**

All of the constructs described in the text are derived from the same ∼4.4 kilobase K10 minigene fragment. The fragment starts at nucleotide (nt) 1 and extends to nt 5363, except the intron has been omitted. Nucleotide positions are from Prost et al. (1988), with sequence corrections described in Cohen and Serano (1995). For further reference, the K10 3'UTR begins at nt 3048 and extends to nt 4499, where the poly(A)-addition recognition sequence (AATAAA) starts at nt 4474.

**Internal deletions of the 3'UTR**

The following constructs contain deletions (deleted segment indicated by numbers) of the 3'UTR: KASS, 3112-3661; KASH, 3112-3970; KASD, 3112-4366; KASHD, 3970-4366; KASH, 3661-3970; KAA, 3661-3749; KAB, 3750-3824; KAC, 3825-3895; KADB, 3896-3970; KADB, 3661-3824; KACD, 3825-3970; KABCD, 3750-3970. The construction of KASS, KASH, KASD, KASHD and KASH was facilitated...
by the presence of naturally occurring SalI (S), Stul (St), HpaI (H) and DraI (D) restriction sites at nts, 3112, 3661, 3970 and 4366, respectively. The KoΔ, Koβ, Koκ, KoΔ, KoΔβ, KoΔκ and KoΔβκ constructs were made by joining appropriate PCR-amplified fragments (details available upon request).

3’ truncations

The K26, KSS26, KSH26, KSSH26 and KSD26 constructs were made by truncating the K10 minigene fragment (described above) at SalI, Stul, HpaI, HpaI and DraI, respectively (coordinates listed above), and thus, lack K10 poly(A)-addition and transcription termination control sequences. Poly(A)-addition and transcription termination control sequences from the hsp26 gene were then spliced onto the truncated minigene fragments. The hsp26 sequences are defined by a 623 nt SalI–ClaI fragment and extend from 317 nt upstream of the hsp26 poly(A)-addition recognition sequence (Southgate et al., 1983). Endogenous hsp26 mRNA is produced in nurse cells, but not transported or localized (Zimmerman et al., 1983). In addition to the 3’ truncation, KSSH26 is internally deleted for the K10 3’UTR SalI–Stul region (nts 3112–3661).

TLS substitution and deletion mutants

Kstem5’, Kstem3’, Kstem3’ D, Kloop and Kbub differ from the 4.4 kb K10 minigene fragment (described above) only in the TLS, as shown in Fig. 7. These mutations were introduced by PCR-mediated, site-directed mutagenesis (details available upon request) and verified by dideoxy-sequencing.

K10-oskar fusion constructs

The K-osk, KoΔ and KoΔ κ constructs were made by truncating the K10 minigene fragment at the SalI site, nt 3112 (see above). Following truncation, osk 3’UTR sequences, with or without the K10 TLS, were added to the 3’ end of the minigene. For reference, the osk 3’UTR corresponds to the region 2551 to ~3593 [nt numbering from Kim-Ha et al., (1991)]. K-osk contains the osk DraI–XbaI fragment (osk nts 2790–3658). The osk 3’UTR sequences of KoΔ and KoΔ κ were generated by deleting the SphI–SacII region (nts 3083–3231) from the ~4 kb SstI–AatII fragment, which extends from nucleotide 2471 to ~3 kb downstream of the osk poly(A)-addition site. In KoΔκ, the osk SphI–SacII fragment is replaced by the K10 TLS (sequence shown in shaded region of Fig. 5A).

Other constructs

KASD+κ and KASD+κ were made by substituting a single PCR-amplified copy of subregion A and a synthetic TLS (sequences given in Fig. 5A), respectively, for the SalI–DraI region (nt 3112–4366) of the K10 minigene. KASH+T was constructed by substituting a single synthetic copy of the TLS for the SalI–HpaI region (nt 3661–3970) of the K10 minigene. KASD+κ was constructed by substituting a single PCR-amplified copy of subregion A (described above) immediately upstream of the hsp26 sequence. 70cSfH26 consists, from 5’ to 3’, of a hsp26–sg3 nurse cell-specific promoter fragment (Frank et al., 1992), all but the first six amino acids of the protein coding region of the E. coli lacZ gene, the K10 SalI–HpaI fragment (nucleotides 3661–3970) and the hsp26 SacII–ClaI fragment (described above).

Transformations

All constructs described above were cloned into the pCaSpeR4 transformation vector (Pirrotta, 1988), with the exception of 70cSfH26, which was cloned into the Germ70 transformation vector (Serano et al., 1994). Constructs were introduced into w1118 flies by P element-mediated transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Transposase activity was provided by the p13wpc helper plasmid (Cohen and Meselson, 1985). Several independently transformed lines were generated and analyzed for each construct, with the exception of KoΔ, in which only one line was analyzed. While there were line to line variations in the amount of mRNA produced by a given construct, no differences were seen in the mRNA distribution patterns. The transgenes were introduced into a K10Lmo mutant background by standard genetic crosses.

In situ hybridization

In situ hybridization to whole-mount ovaries was carried out according to Tautz and Pfeifle (1989) with modifications described in Cheung et al. (1992). Digoxigenin-labeled DNA probes were produced by random-priming according to Feinberg and Vogelstein (1983). The K10 probe corresponds to nucleotides 757–1763 (Prost et al., 1988). The lacZ probe was derived from sequences in the pMC1871 plasmid (Pharmacia; see also Serano et al., 1994). In situ hybridization to mRNA produced by K10 transgenes was carried out in a K10Lmo background, which produces no detectable endogenous K10 mRNA (Cheung et al., 1992). Photography was carried out as previously described (Serano et al., 1995).

RESULTS

In previous studies, we mapped K10 mRNA transport/localization control sequences to the ~1400 nucleotide (nt) 3’UTR of the mRNA (Cheung et al., 1992). To delimit further the K10 mRNA transport/localization control sequences, we constructed a series of K10 deletion mutants lacking various portions of the K10 3’UTR. Unless otherwise noted, each deletion was made within the context of the same 4.4 kilobase K10 minigene that we and others have previously shown possesses wild-type K10 gene activity in transgenic flies (Haenlin et al., 1987; Serano and Cohen, 1995). The transgenes were introduced into a K10Lmo mutant background which produces no endogenous K10 mRNA (Cheung et al., 1992), and tested for their ability to produce transported/localized mRNA by in situ hybridization.

The K10 poly(A)-addition and transcriptional termination control regions are not required for K10 mRNA transport/localization

We first tested whether the K10 poly(A)-addition and putative transcription termination control regions are required for mRNA transport/localization. We made a K10 construct, called KSD26, in which the 3’ end of the K10 gene, starting at a site 108 nt upstream of the poly(A)-addition control site (AATAAA), was replaced with the poly(A)-addition and transcription termination control sequences from the 3’ end of the hsp26 gene. As seen in Fig. 2B, the KSD26 mRNA distribution pattern is indistinguishable from that of wild-type K10 mRNA. The mRNA is transported to the posterior pole of the oocyte during stages 1-7 and subsequently localized to the oocyte’s anterior cortex during stages 8-10B. In contrast, a control transgene, called K26, which contains the same hsp26 3’ end fragment, but lacks all but the first 65 nt of the K10 3’UTR (Fig. 1), produces mRNA that is retained in nurse cells until nurse cell regression, i.e., until the indiscriminate transfer of nurse cell cytoplasm to the oocyte at stage 11 (Fig. 2C). We conclude that the K10 poly(A)-addition site and putative transcription termination control sequences are not required for K10 mRNA transport/localization.

While K10 transgenes containing hsp26 poly(A)-addition and putative transcriptional termination control sequences
produce efficiently transported and anteriorly localized mRNA, they exhibit reduced gene activity compared to their wild-type counterparts; K10 mutants carrying one copy of the KSD26 transgene produce eggs that hatch with a frequency of ~20-80%, while K10 mutants that carry one copy of a K10 transgene containing an intact 3' end typically produce eggs that hatch at a frequency of ≥95% (data not shown). Based on immunocytochemical analyses (data not shown), we attribute the reduced activity of the KSD26 transgene to inefficient translation of its mRNA. Importantly, the distribution pattern of protein produced from KSD26 mRNA is indistinguishable from that of endogenous K10 mRNA (data not shown).

The 309 nt Stu-I-HpaI region is necessary and sufficient for K10 mRNA transport/localization

We next analyzed a series of transgenes lacking different internal portions of the K10 3'UTR. As summarized in Fig. 1, these studies identify a 309 nt Stu-I-HpaI fragment that is both necessary and sufficient for K10 mRNA transport/localization. This is best illustrated by two constructs: KASiH and KStH26. KASiH specifically lacks the Stu-Hpa region and produces transcripts that are retained in nurse cells until nurse cell regression (Fig. 2D). In contrast, KStH26, which contains the

![Fig. 1. Low-resolution mapping of K10 mRNA transport/localization control sequences. All deletions (with the exception of 70zStH26) were created in the context of a K10 minigene fragment possessing wild-type K10 gene activity (see Materials and Methods). The constructs differ only in the region shown. The black lines represent K10-derived sequences, where deletions are represented by gaps in the line. The approximate positions of the K10 translation stop codon (TAG) and poly(A)-addition site (pA) are indicated. The bottom six constructs contain hsp26-derived poly(A)-addition and putative transcription termination control sequences (labeled rectangle). The ability of the transgenes to produce transported/localized mRNA, as determined by in situ hybridization, is summarized to the right of the constructs (see also Fig. 2). The transcriptional control and protein coding regions of the 70zStH26 construct consists of the hsp26 nurse cell enhancer with an adjoining Sgs3 promoter followed by the E. coli lacZ protein coding region. S, SalI; St, StuI; H, HpaI; D, DraI; P, PstI; WT, wild type.](image)

![Fig. 2. Whole-mount in situ hybridization of K10-deletion and K10-hsp26 fusion genes. Egg chambers are arranged from left to right in order of increasing stage of development following whole-mount in situ hybridization with a digoxigenin-labeled K10 (A-E) or lacZ (F) probe. Egg chambers carry the following transgenes: (A) none (wild-type control); (B) KSD26; (C) K26; (D) KASiH; (E) KStH26; (F) 70zStH26. Transgenes listed in B-E are in a K10LM00 background, which does not produce any detectable endogenous K10 mRNA (Cheung et al., 1992). In this and all other figures, anterior is to the left.](image)
**Stu-HpaI** region but lacks all other portions of the 3′UTR (except the first 65 nt), produces transcripts that are transported and anteriorly localized within the oocyte (Fig. 2E). We conclude that most, if not all, of *K10*’s transport/localization control sequences are contained within the 309 nt *Stu-HpaI* region.

To test whether the *Stu-HpaI* region is sufficient to direct the transport/localization of a heterologous mRNA, we constructed 70zStH26. The only *K10* sequence contained in this *lacZ*-tagged transgene is the 309 nt *Stu-HpaI* region (Fig. 1). As seen in Fig. 2F, 70zStH26 mRNA is efficiently transported and localized. We conclude that the 309 nt *Stu-HpaI* region is sufficient to direct the transport and anterior localization of a heterologous mRNA.

**A 92 nt subregion of *Stu-HpaI* is necessary and sufficient for *K10* mRNA transport/localization**

To map more precisely *K10* mRNA transport/localization control sequences, we conceptually subdivided the 309 nt *Stu-HpaI* region into four non-overlapping subregions, denoted A-D. We deleted each subregion individually, or in combination, within the context of an otherwise intact *K10* 3′UTR. As summarized in Fig. 3A, our analysis of these constructs indicates that *K10* mRNA transport/localization control sequences are fully contained within subregion A. When we delete subregion A (see e.g., KΔA), only non-transported/non-localized mRNA is produced (Fig. 4A). Conversely, *K10* transgenes lacking subregions B, C and/or D (see e.g., KΔBCD) produce efficiently transported/localized mRNA (Fig. 4B). These results show that subregion A is required for *K10* mRNA transport/localization and that subregions B-D are dispensable.

To test whether subregion A is sufficient for mRNA transport/localization, we constructed KASD+A and KA26 (Fig. 3B). The 3′UTRs of these transgenes contain the first 65 nt of the *K10* 3′UTR followed by a single copy of subregion A. The two constructs differ in that the poly(A)-addition site and putative transcription termination control regions of KASD+A and KA26 are derived from the *K10* and *hsp26* genes, respectively. Constructs identical to KASD+A and KA26, but lacking subregion A, produce transcripts that are not transported or localized (see KΔA and K26 in Fig. 1). As seen in Fig. 4C (and data not shown), KASD+A and KA26 produce mRNA that is transported and anteriorly localized. We conclude that most, if not all, of *K10*’s mRNA transport/localization control sequences are contained within subregion A. We cannot, however, rule out the possibility that there are redundant copies of a second essential sequence, one or more in the region extending from the first nt of *K10* mRNA to 65 nt downstream of TAG, and one or more within subregions B-D.

**The identification of the 44 nt *K10* mRNA transport and localization sequence (TLS)**

Upon visual inspection of the 92 nt subregion A, we identified a 44 nt sequence that has a potential to form a stem-loop...
The TLS is sufficient for the transport and anterior localization of recombinant osk transcripts, but overrides osk posterior localization control sequences

We next wanted to see if the TLS could substitute for the transport/anterior localization control region of a mRNA whose final destination within the oocyte is different than K10’s. We chose osk, since its mRNA transport and localization control sequences had been previously defined. Specifically, Kim-Ha et al. (1993) identified a 150 nt Spf1-SacII region that mediates osk mRNA transport and a separate region that mediates late stage posterior localization. We first replaced K10’s 3’UTR with osk 3’UTR sequences to determine whether osk transport and localization control sequences retain activity when fused to K10 protein coding sequences and produced under the transcriptional control of the K10 promoter/enhancer. This control construct, called K-osk, produces mRNA that, like wild-type osk, is transported to the oocyte’s posterior pole during stages 1-7, transiently localizes to the oocyte’s anterior cortex during early stage 8, and moves back to the oocyte’s posterior pole during late stage 8/early stage 9, where it persists through the remainder of oogenesis (Fig. 6A,B and data not shown; Kim-Ha et al., 1993; Ephrussi et al., 1991).

We then constructed two K10-oskar hybrid transgenes, called KoΔ and KoΔ+T, that lack osk mRNA transport sequences, but retain osk posterior localization sequences (Fig. 6A). In KoΔ+T, the deleted osk transport control sequences are replaced with the K10 TLS, while in KoΔ there is no such replacement. As seen in Fig. 6B, KoΔ mRNA is distributed in a manner similar to that reported for other osk mRNAs that lack osk transport control sequence, but retain posterior localization control sequences (see ocl15 in Kim-Ha et al., 1993). KoΔ transcripts are detected in nurse cells through early stage 8. Beginning at the end of stage 8, and extending through the end of stage 10B, low amounts of KoΔ transcripts are also seen at the oocyte’s posterior pole. Presumably, low amounts of KoΔ transcripts diffuse from nurse cells into the oocyte during early stages of oogenesis and become detectable in the oocyte after stage 8 as they concentrate at the posterior pole via the intact posterior localization control sequences. In contrast to KoΔ mRNA, KoΔ+T mRNA is efficiently transported into the oocyte during early stages of oogenesis (Fig. 6B). However, while K-osk and wild-type osk mRNAs accumulate only transiently at the oocyte’s anterior cortex during stage 8, KoΔ+T mRNA remains there through at least stage 10B (Fig. 6B; detection becomes problematic after stage 10B due to the secretion of the egg chorion). We conclude that the K10 TLS can direct the transport of a heterologous mRNA. In addition, the K10 TLS apparently overrides the activity of osk mRNA posterior localization control sequences.

The stem-loop secondary structure of the K10 TLS is apparently essential for TLS function

As mentioned above, the K10 TLS was originally identified by its striking potential to form a stem-loop secondary structure. To determine whether TLS activity is dependent on such a
structure, we introduced small mutations into the TLS, designed to disrupt its predicted secondary structure and primary sequence, or only its primary sequence.

The first two constructs, \(K_{stem5}'\) and \(K_{stem3}'\), contain mutations that disrupt the TLS primary sequence as well as its putative stem-loop structure. In \(K_{stem5}'\), we replaced five internal uracil residues on the 5' side of the stem with five adenine residues (Fig. 7). Reciprocally, in \(K_{stem3}'\), we replaced five internal adenine residues on the 3' side of the stem with five uracil residues (Fig. 7). As seen in Fig. 7, \(K_{stem5}'\) and \(K_{stem3}'\) mRNAs are neither transported nor localized. In addition, \(K10\) mutants carrying one copy of either \(K_{stem5}'\) or \(K_{stem3}'\) produce eggs that typically hatch with a frequency of \(\leq 10\%\) (data not shown). Thus, two separate five nucleotide substitutions in the TLS block its ability to direct mRNA transport/localization, even in the context of an otherwise wild-type \(K10\) transgene. These results show that the TLS is essential for \(K10\) function and mRNA transport/localization.

The uracil-to-adenine and adenine-to-uracil substitutions described above could block transport/localization because they alter the TLS primary sequence or because they alter the predicted stem-loop structure. To distinguish between these possibilities, we combined the uracil-to-adenine substitutions of \(K_{stem5}'\) with the compensatory adenine-to-uracil substitutions of \(K_{stem3}'\) to make \(K_{stem53}'\) (Fig. 7). \(K_{stem53}'\) thus carries the primary sequence lesions of both \(K_{stem5}'\) and \(K_{stem3}'\), but has the potential to form the same stem-loop structure predicted for wild-type TLS. As seen in Fig. 7, \(K_{stem53}'\) mRNA is transported/localized almost as efficiently as is wild-type \(K10\) mRNA. Moreover, \(K_{stem53}'\) transgenes restore almost complete fertility to \(K10\) mutant stocks. \(K10\) mutants that carry one copy of the \(K_{stem53}'\) transgene produce eggs that typically hatch at a frequency of \(\geq 80\%\) (data not shown). These data indicate that extensive alterations of the TLS primary sequence have little or no adverse affect on TLS transport/localization activity, provided that they do not disrupt the predicted stem-loop secondary structure. We conclude that TLS activity is likely to be dependent on the formation of the predicted stem-loop secondary structure.

While the above experiments provide strong evidence that TLS activity is dependent on an encoded stem-loop secondary structure, they do not provide much insight into the nature of the interactions between the TLS and the transport/localization machinery. Given the selectivity of mRNA transport and localization, it is likely that such interactions include base-specific recognition events (St Johnston, 1995). Because the major groove of double-stranded RNA is deep and narrow (Alden and Kim, 1979; Seeman et al., 1976), we thought it likely that base-specific interactions would be restricted to the single-stranded regions of the predicted stem-loop secondary structure. To look for such interactions, we made \(Kloop\) and \(Kbub\). In \(Kloop\), we introduced transition mutations into all eight positions of the predicted TLS loop (Fig. 7). In \(Kbub\), we removed the two nucleotides that bubble out from the 3' side of the predicted stem (Fig. 7). As seen in Fig. 7, \(Kloop\) and \(Kbub\) both produce transported/localized mRNA, although like \(K_{stem53}'\), the transport/localization and rescue activities of \(Kloop\) and \(Kbub\) mRNA are slightly less efficient than wild type (Fig. 7 and data not shown). These results indicate that the predicted single-stranded regions of the TLS do not make essential base-specific contacts with the transport/localization machinery. The possibility that other regions of the predicted stem-loop structure, viz., the stem termini, are engaged in such base-specific recognition events is discussed below.

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**Fig. 6.** Schematic diagrams and mRNA distribution patterns of \(K10-oskar\) hybrid transgenes. (A) The thick black lines represent \(K10\)-derived sequences. The thick gray lines represent \(osk\)-derived sequences. The interruption in the line represents the deletion of the ~150 nt \(SphI-SacII\) region, which contains \(osk\) mRNA transport control sequences (Kim-Ha et al., 1993). The small stem-loop represents the 44 nt \(K10\) TLS (sequence shown in Fig. 5). The ability of the transgenese to produce transported mRNA (transport) and the final destination of the mRNA within the oocyte (destination within oocyte) are indicated to the right of the diagrams. (B) In situ hybridization montages showing the distribution patterns of \(K-osk\), \(Ko\Delta\) and \(Ko\Delta+T\) mRNA (see Fig. 2 legend for in situ hybridization details).
DISCUSSION

A single element mediates K10 mRNA transport and anterior localization

We have identified a 44 nt sequence, the TLS, which is essential for K10 gene function and is necessary and sufficient for mRNA transport into, and anterior localization within, the Drosophila oocyte. The small size of the TLS suggests that it acts as a single element to direct a single transport/localization event. Indeed, both K10 mRNA transport to the posterior pole of the oocyte during stages 1-7 and localization to the oocyte’s anterior cortex during stages 8-10B could be mediated by the continuous association of the mRNA with a minus end-directed microtubule motor (see Cooley and Theurkauf, 1994).

Since most mRNAs that are localized within the Drosophila oocyte are transported into the oocyte during stages 1-7 and accumulate at least transiently at the oocyte’s anterior cortex during the early part of stage 8, one might expect many of them to contain the TLS. We have identified one such candidate sequence in orb mRNA (Fig. 8; Lantz et al., 1992). The orb sequence and the K10 TLS are quite similar in A-U content and in the size of the predicted stem and loop. In addition, the

Fig. 7. The distribution patterns of mRNA produced from K10 transgenes containing TLS-mutations. The TLS primary sequence and predicted secondary structure is shown for wild type, Kstem5’, Kstem3’, Kstem5’3’, Kloop and Kbub (nucleotide substitutions represented by lowercase letters). To the right of each sequence/structure are egg chambers carrying the respective transgene in a K10Δ3400 background following whole-mount in situ hybridization with a digoxigenin-labeled K10 probe.

Fig. 8. A comparison of the predicted secondary structures of the K10 TLS and a sequence found within the orb 3’UTR. A region of particularly strong homology is found near the loop of both predicted stems (boxed region). The orb sequence corresponds to nucleotides 4200-4241 (according to Lantz et al., 1992) and was initially identified by visual inspection. We also searched Adducin-like, bcd, Bicaudal-D, cyclin B, gurken, Hsp83, nanos, osk, pumilio, staufen, tudor and yemanuclein-a mRNA sequences using the sequence W7N7oW7, where W’ represents base complementary to W. A single match was identified in both Adducin-like and nanos, but such sequences showed little or no primary sequence homology with the TLS (not shown). No other matches were found.
orb sequence resides in a 280 nt region of orb mRNA that is sufficient for orb mRNA transport and anterior localization (Lantz and Schedl, 1994). These findings, together with the similarities of K10 and orb mRNA distribution patterns through stage 11 (Cheung et al., 1992; Lantz et al., 1992), suggest that K10 and orb mRNAs are recognized by the same component(s) of the transport/localization machinery. We have been unable to identify candidate TLSs in any other mRNAs known to be localized within the Drosophila oocyte (see Fig. 8 for details). This raises the possibility that the transport of at least some mRNAs is mediated by factors different than those that mediate K10, and possibly orb. We cannot rule out, however, that other localized mRNAs share a common secondary structure motif with the TLS that is not easily identifiable by a comparison of primary sequences.

While most localized mRNAs appear to be transported into the oocyte in a similar fashion, our results suggest that the mRNA transport control sequences of different mRNAs are not functionally equivalent. For example, the K10 TLS can substitute for osk mRNA transport control sequences, but it is apparently incompatible with the activity of osk mRNA posterior localization control elements. The observed incompatibility may occur for superficial reasons, e.g., the inclusion of the TLS in the osk 3’UTR may alter the secondary structure of osk posterior localization control sequences. Alternatively, osk mRNA transport control sequences, unlike the K10 TLS, may facilitate the release of the mRNA from a minus end-directed microtubule motor at stage 8, thus allowing osk posterior localization control sequences to mediate the association of osk mRNA with a plus end-directed microtubule motor.

mRNA transport and localization control elements and RNA secondary structure

The TLS has a predicted secondary structure consisting of a 8 base loop and an imperfect, 17 base pair stem – one strand of the stem contains two one-base bubbles (Fig. 5B). Consistent with the idea that TLS activity is dependent on the formation of such a stem-loop structure, we find that TLS transport/localization activity is abolished when base changes are introduced into one of the two stem strands alone, but not when simultaneously introduced into both strands in a compensatory manner that preserves the predicted secondary structure. Other mRNA transport/localization control sequences have also been proposed to form secondary structures. Most notably, the 625 nt bcd mRNA localization signal is predicted to form an elaborate secondary structure, consisting of five large stems (Macdonald and Struhl, 1988; Macdonald, 1990; Ferrandon et al., 1994). This is supported by the finding that, while the primary sequence of the bcd localization signal varies significantly between a number of Drosophila species, the putative secondary structure remains conserved (Macdonald, 1990). In addition, a staufer protein, which mediates bcd and osk mRNA localization, exhibits double-stranded RNA-binding activity in vitro (St. Johnston et al., 1992). Together, these findings suggest that secondary structure formation is a common feature of mRNA transport/localization control sequences.

Given the selective nature of mRNA transport/localization, it is likely that recognition of the TLS by the transport/localization machinery requires base-specific contacts. A complete base substitution of the predicted loop, or the removal of the two single nucleotide bubbles from the 3’ side of the stem, have only a slight negative affect on TLS activity, indicating that these regions do not provide essential base-specific contacts. It could be that residues of the stem itself make base-specific contacts with the transport/localization machinery. The minor groove of double-stranded RNA is wide and accessible, but it contains few hydrogen bond acceptor and donor groups and the potential for base-specific hydrogen bonding is very low (Seeman et al., 1976). One possible base-specific hydrogen bond acceptor/donor group in the minor groove is the carbonyl group of any uracil immediately 3’ to adenine (Rosenberg et al., 1973). The TLS stem contains two such groups. The predicted orb stem (Fig. 8) also contains several such groups, but at non-conserved positions. Assuming that the overall conservation between the predicted stem loop structures of K10 and orb is not fortuitous, it would thus appear that the minor groove does not play a major role in the base-specific recognition of the stem by the transport/localization machinery. In contrast to the minor groove, the major groove of double-stranded RNA has a rich, base-specific collection of hydrogen bond acceptor and donor groups (Seeman et al., 1976). While the major groove of double-stranded RNA is narrow and generally not accessible to small, amino acid-size molecules (Alden and Kim, 1979), chemical acylation studies show that bases positioned near a loop or bubble of two or more nucleotides are accessible, presumably due to a local distortion of the helix (Weeks and Crothers, 1993). Accessibility extends into the duplex about 2 nucleotides on one strand and about 4 nucleotides on the other. The bases adjacent to the TLS loop are conserved in orb (Fig. 8) and thus are good candidates for base-specific interactions with the transport/localization machinery. Residues at the base of the stem are not conserved and thus apparently less likely to make specific contacts with the transport/localization machinery.

The protein components of the transport/localization machinery have remained elusive. Genetic analyses have identified only two genes, Bic-D and egalitarian (egl), that are required for K10 mRNA transport into the oocyte (Suter and Steward, 1991; Cheung et al., 1992). However, it is unclear whether Bic-D and egl encode proteins that bind directly to the TLS. These genes are also required for oocyte determination and microtubule organization, developmental events that are likely to be a prerequisite for mRNA transport (Schüpbach and Wieschaus, 1991; Theurkauf et al., 1993). Thus, biochemical approaches may be most useful for identifying the proteins that directly contact the K10 TLS.

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