Identification of cis Elements Which Direct the Localization of Maternal mRNAs to the Posterior Pole of Ascidian Embryos

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During ascidian embryogenesis, some mRNAs show clear localization at the posterior-most region. These postplasmic mRNAs are divided into two groups (type I and type II) according to their pattern of localization. To elucidate how these localization patterns are achieved, we attempted to identify the localization elements of these mRNAs. When in vitro synthesized postplasmic mRNAs were introduced into eggs, these mRNAs showed posterior localization similar to the endogenous mRNAs. The posterior localization of these mRNAs was mediated by their 3′ untranslated regions (3′ UTRs), as is the case for several localized Drosophila and Xenopus mRNAs. We identified smaller fragments of the 3′ UTRs of HrWnt-5 and HrPOPK-1 mRNAs (type I) and HrPet-3 mRNA (type II) that were sufficient to direct green fluorescent protein mRNA to the posterior pole. For the localization of HrWnt-5 mRNA, two UG dinucleotide repetitive elements were essential. Motifs similar to these small elements also exist within the HrPOPK-1 mRNA localization element and 3′ UTR of HrZF-1 mRNA, suggesting the conservation of localization elements among type I mRNAs. In contrast, the smallest sequence that suffices for the posterior localization of HrPet-3 (a type II mRNA) has different features from those of type I mRNAs; indeed, it does not have an identifiable critical element. This difference may distinguish type II mRNAs from type I mRNAs. These findings, especially the identification of the small localization element of HrWnt-5 mRNA, provide new insights into the localization of mRNAs during ascidian embryogenesis.

Key Words: ascidians; posterior; localized mRNA; type I; type II; localization element.

INTRODUCTION

The ascidian egg is one of the best examples of a mosaic egg because the mosaicism can be seen based on color differences of the cytoplasm (Conklin, 1905a,b). Embryological experiments have provided evidence about the mosaicism of ascidian eggs by showing that the determinants of the differentiation of tissues, axis formation, and gastrulation are localized in specific regions of fertilized eggs (Bates and Jeffery, 1987; Jeffery, 1991; Nishida, 1992, 1993, 1994a,b). Of particular note, the myoplasm, the distinctive cytoplasm that forms a crescent at the posterior–vegetal region of fertilized eggs, is distributed exclusively to the muscle precursor cells, and if myoplasm is artificially distributed to other lineages of cells, these cells change their fate to muscle lineage (Whittaker, 1982; Deno and Satoh, 1984; Nishida, 1992). Based on the clear correlation between the distribution and function of myoplasm, the existence of muscle determinants in the myoplasm was evident. Although most such determinants have not yet been identified, several reports support the idea that localized maternal RNA is one candidate for such a determinant (Jeffery, 1990a,b; Marikawa et al., 1995). Recent studies on maternal mRNAs have led to the identification of many mRNAs that are localized in the posterior–vegetal region of eggs and embryos (Yoshida et al., 1996; Satou and Satoh, 1997; Satou, 1999; Sasakura et al., 1998a,b, 2000; Makabe et al., 2001; Nishikata et al., 2001). The localization pattern of these mRNAs is very similar to that of myoplasm. Based on this similarity, we speculate that these posteriorly localized mRNAs share some features with myoplasm with respect to both function and the localization pathway. Regarding function, Nishida and Sawada (2001) have shown that the depletion of macho-1 mRNA, which is also localized at the posterior pole, caused complete loss of muscle cells in...
Halocynthia embryos, suggesting that macho-1 mRNA is a muscle determinant. Regarding the localization pathway, the localization of some of these mRNAs has been shown to be dependent on the same cytoskeletal components as myoplasm (Zalokar, 1974; Sawada and Osanai, 1981; Yoshida et al., 1998; Sasakura et al., 2000). However, many questions remain unsolved about the localization mechanisms. For example, how are these mRNAs selected by localization machinery from the total population of diverse maternal mRNAs? What machinery of the cytoplasm links these mRNAs to the cytoskeleton, and how does this machinery carry mRNAs to the posterior region? Solving these problems will be necessary to achieve complete understanding of how ascidian mosaic eggs are created and how the embryogenesis is regulated by determinant mRNAs.

During the course of studying mRNA localization, we found that the pathway for localization of mRNAs at the posterior pole is not so simple. There are two independent pathways, although the final destination is the same. In a previous report, we showed that these “postplasmic” mRNAs (i.e., RNAs localized at the posterior pole) can be divided into two groups according to the timing of the localization (Sasakura et al., 2000). Type I mRNAs are localized at the cortex of unfertilized eggs, and then are transported to the posterior pole during the first and second ooplasmic segregations after fertilization. This pattern of localization is the same as that of myoplasm (Conklin, 1905b; Sawada, 1988; Sardet et al., 1989). In contrast, the mRNAs of the other group, the type II mRNAs, are uniformly distributed in unfertilized eggs. Nevertheless, a fraction of the type II mRNAs is transported to the posterior pole during two phases of the ooplasmic segregation. In spite of the accumulation during ooplasmic segregations, the localized signal of type II mRNAs was rarely detected at the two-cell stage. Type II mRNAs are sequestered again at the posterior pole during the second cleavage stage. The second migration of the type II mRNAs enabled us to clearly observe localized signals at the posterior pole. By using cytoskeleton-depolymerizing drugs, we have shown that the localization of both type I and type II mRNAs depends on actin filaments and microtubules, but in different ways (Sasakura et al., 2000). This finding raised another question: how is the different localization pattern of type I and type II mRNAs established although both types of mRNA use the same cytoskeleton? In order to address this question, it will be necessary to identify and compare the localization element(s) of both type I and type II mRNAs and RNA-binding protein(s) that link these mRNAs to the cytoskeletal elements.

The identification of localization elements of ascidian mRNAs will solve questions about their evolutionary relationships. In many organisms, most mRNA localization is mediated by the 3’ UTR, a fact that has been widely observed and now is almost accepted as a generality (reviewed in Micklem, 1995; Bashirullah et al., 1998; Kloc et al., 2002). However, in the study of early embryogenesis, it has been confirmed in only a few organisms. To generalize this principle, it is essential to determine its validity in other species. In vertebrates, Xenopus provides good material for the study of RNA localization, and in fact, much of our knowledge of mRNA localization has been achieved by studies in Xenopus (reviewed in King et al., 1999; Kloc et al., 2002). For Vg1 mRNA localization, two trans-acting factors, vera and Vg1-RBP, were isolated, and both of them are homologs of Zipcode-binding protein (ZBP), a protein that functions in the localization of β-actin mRNA of chick fibroblast cells (Ross et al., 1997). This cross-species conservation is interesting, and it should be determined whether such a conserved system of localization is used globally among vertebrates and their ancestor chordates. The localization element of Vg1 mRNA has been analyzed in detail. Therefore, a comparison between the localization elements of Vg1 and other localized mRNAs of chordates would likely be revealing. Ascidians, one of the most primitive chordates, are an ideal experimental system, because the localization of several of their mRNAs is clearly evident and has been described well. In addition, some techniques that are essential for the study of RNA localization, including the microinjection into eggs, are already established for ascidians.

In this study, we found that exogenous mRNAs injected into unfertilized eggs were correctly localized at the posterior pole in the same way as their endogenous counterparts. Using this system, we showed that the posterior localization of maternal mRNAs is mediated by their 3’ UTRs, as has been reported in other organisms (reviewed by Micklem, 1995; Bashirullah et al., 1998). For HrWnt-5, HrPOPK-1 (type I), and HrPet-3 mRNAs (type II), a portion of the 3’ UTR was sufficient for the RNA localization. We have identified two UG dinucleotide-repetitive elements (UGREs), both of which are required for the localization of HrWnt-5 mRNA. Similar UGREs also exist in the HrPOPK-1 mRNA localization-sufficient sequence and the 3’ UTR of HrZF-1 mRNA. Therefore, it is possible that the UGRE is the consensus localization element of type I mRNAs. The features of the localization element of HrPet-3 mRNA are distinct from those of type I mRNAs: HrPet-3 mRNA does not have one specific element indispensable for localization. Rather, our data suggest the existence of multiple localization elements within the localization sequence of HrPet-3 mRNA that may act redundantly for the localization. These differences may contribute to the differences of the binding to the cytoskeletal components of type I and type II mRNAs that result in the differential movement of mRNAs toward the posterior pole.

MATERIALS AND METHODS

Biological Materials

Halocynthia roretzi (Urochordata, Tunicata) was purchased from fishermen near the Asamushi Marine Biological Station, Tohoku University, Aomori or the Ohtsuchi Marine Research
Center of the Ocean Research Institute, University of Tokyo, Iwate, Japan, during the spawning season. H. roretzi is a hemaphroditic and is self-sterile. Naturally spawned eggs were fertilized with a suspension of nonself sperm. After insemination, eggs were reared at approximately 11°C in Millipore-filtered sea water (MFSW) containing 50 µg/ml streptomycin.

 Constructs

Full-length type I and type II cDNAs were cloned into the multicloning site of pBluescript RN3 (Lemaire et al., 1995). The 3' UTRs were deleted from these constructs to prepare 5' UTR + ORF constructs. The GFP (Clontech) open reading frame (KpnI–EcoRI fragment; the KpnI site was blunt ended) was subcloned into the pBluescript RN3 BglII (blunt-ended)–EcoRI site (pRN3 GFP-NLS). The 3' UTRs of the cDNAs of the type I and type II mRNAs (cloned into pBluescript SK–) were cut off by restriction enzymes, and after the ends were blunt, the fragment was subcloned into the blunt-ended NotI site of pRN3 GFP-NLS. The restriction enzymes used for cutting the 3' UTR were HindIII/Xhol for HrWnt-5, EcoT221/Xhol for HrPOPK-1, EcoRV/Xhol for HrZF-1, Xhol for HrPet-1, EcoT221/Xhol for HrPet-2, and AffII/Xhol for HrPet-3.

For the restriction enzyme-mediated deletion of the 3' UTR, the 3' UTRs of HrWnt-5, HrPOPK-1, and HrPet-3 mRNAs subcloned into pRN3 GFP-NLS were cut by restriction enzymes, and the products were self-ligated after the blunt-terminating reaction. The pairs of restriction enzymes used were SacI/EcoRI, EcoT221/EcoRI, EcoRV/EcoRI, SacI/BstEII, EcoT221/BstEII, and EcoRV/BstEII for HrWnt-5, Spel/EcoRI, MunI/EcoRI, BglII/EcoRI, Spel/BstEII, MunI/BstEII, and BglII/BstEII for HrPOPK-1, and EcoRI, HindIII/EcoRI, HindIII/BstEII, BglII/BstEII, and BglII/HindIII for HrPet-3. Small deletions of the HrWnt-5 3' ET fragment and the HrPet-3 3' H fragment were made by using PCR, and the PCR products were subcloned into the blunt-ended NotI site of pRN3 GFP-NLS.

Substitution of the nucleotide sequence of the HrWnt-5 3' ET/3' d60 fragment and HrPet-3 3'LS fragment was carried out by PCR. The sequence used for nucleotide substitution was 5'-tagcattcgatgtctgcctc-3' (a part of the Xenopus β-globin UTR). In the case of HrWnt-5 R1, 5'-tagcattcgatgtctgcctcataa-3' was used. The correct substitution of nucleotides was checked by sequencing analysis. When a one-base gap was observed, it was filled by using the PCR as a template and with 5'-tagcattcgatgtctgcct-3' and 5'-ggagcagataccgaaggtg-3' primers.

For the preparation of 2°R1 constructs, R1for and R1rev oligonucleotides (R1for: 5'-gtcttagaatgttttgatgttaatttggc-3'; R1rev: 5'-gaagaaaaattacacacaaaaa-3') were annealed in annealing buffer (100 mM Tris–HCl, pH 7.4, 70 mM NaCl), and then gaps were filled in using T4 DNA polymerase (NEB). Annealed R1 was cut by EcoRI and inserted into the pBluescript EcoRI/EcoRV site (pBSR1). Next, annealed R1 was cut by Xbal and inserted into the Xbal/Smal site of pBSR1 (pBS2°R1). 2°R1 was amplified by PCR using pBS2°R1 as template and T3 and T7 primers, and the product was then cut by NotI/HindIII and inserted into the NotI/SacI site (the SacI site was made blunt-ended) of pRN3 GFP-NLS.

Preparation of mRNA and Microinjection of mRNA into Unfertilized Eggs

Constructs prepared as described above were linearized by cleavage with restriction enzyme Asp718 or PstI (when an Asp718 site existed within the insert). Because pBluescript RN3 contains the 5' and 3' UTRs of Xenopus β-globin mRNA, all of the mRNAs used in this study were conjugated with the 5' and 3' UTRs of Xenopus β-globin mRNA at the two ends to protect the mRNAs from degradation. Digoxigenin-labeled mRNAs were synthesized by using T3 RNA polymerase and a digoxigenin RNA labeling mix (Roche). Approximately 4–5 digoxigenin groups per 100 nucleotides were added. Synthesis of full-length mRNA was checked by electrophoresis. After synthesis, template DNA was degraded by DNase I. The synthesized mRNA was ethanol precipitated and dissolved again in water. One microliter of the solution was used for checking the labeling efficiency by dot blotting. mRNA was mixed with Fast Green dye (final concentration 2 µg/µl) and stored at −80°C until use. A concentration of about 0.1 µg/µl of mRNA in the injected samples was adequate for detecting the mRNA localization.

Microinjection of RNA into unfertilized eggs was carried out as described by Nishida and Sawada (2001), except that we used water instead of a KCl solution for the injection buffer, as mentioned above. The typical injection volume was about 150 pl per egg.

Detection of Exogenous mRNA

Embryos at the indicated stages were fixed with 4% formaldehyde in 0.5 M NaCl and 0.1 M Mops (pH 7.5) overnight at 4°C. Fixed embryos were washed with PBST (phosphate-buffered saline containing 0.1% Tween 20) four times, and incubated with 5 µg/ml Proteinase K in PBST for 30 min at 37°C. After embryos were washed twice with PBST, they were postfixed with 4% formaldehyde in 0.5 M NaCl and 0.1 M Mops (pH 7.5) for 1 h at room temperature. After embryos were washed four times with PBST, they were blocked with a 0.5% solution of blocking reagent (Roche) in PBST for 1 h at room temperature, and then incubated for 2 h with alkaline phosphatase-conjugated antibody against digoxigenin (Roche) diluted 1:2000 with PBST at room temperature. After the embryos were washed with PBST (15 min, 4 times), they were washed once with alkaline phosphatase buffer (100 mM NaCl, 50 mM MgCl2, 100 mM Tris–HCl, pH 9.5), and then signals were detected by using NBT and X-phosphate. Reactions were stopped by PBST, and signals were observed. Embryos that had easily recognizable signals were scored as strong localization. Some embryos were dehydrated and rendered transparent with a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate (BABB) for photographing.

RESULTS

The Exogenous RNAs of Type I and Type II mRNAs Are Localized at the Posterior Pole

Microinjection of labeled mRNAs and checking their localization is an easy and powerful way to identify the localization element of localized mRNAs, if it can be verified that the labeled mRNAs show identical localization to the corresponding endogenous ones. We first tested whether the labeled exogenous RNAs can localize correctly in ascidian embryos. One problem we needed to address was when to introduce the exogenous mRNAs into eggs. Microinjection into fertilized eggs of H. roretzi would not be suitable for our aim, because both types of postplasmic mRNAs start to become localized just after fertilization (Sasakura et al., 2000). Therefore, we decided to introduce...
mRNAs into unfertilized eggs. HrWnt-5 (type I mRNA) or HrPet-3 (type II mRNA) full-length mRNAs were injected into unfertilized eggs. After fertilization, embryos were fixed at the eight-cell stage (the stage at which the posterior pole of embryos can easily be distinguished), and then exogenous mRNA was detected. As shown in Figs. 1A and 1B, both the HrWnt-5 and HrPet-3 exogenous mRNAs were clearly localized at the posterior pole of the embryos. The localization position was the same as that of the corresponding endogenous mRNAs (Sasakura et al., 1998a, 2000), suggesting that correct localization occurred via the normal localization pathway. Uninjected control embryos did not show any signals, indicating that the anti-digoxigenin antibody did not cross-react with unknown epitopes (Fig. 1D). To show that the localization of exogenous mRNAs is specific for mRNAs that are endogenously localized, Gfp and lacZ mRNAs were injected. Neither of these mRNAs was localized at all (Fig. 1C, and data not shown), suggesting that localization of exogenous mRNAs is specific to the nucleotide sequence of endogenously localized mRNAs. To test whether exogenous mRNAs of other type I or type II mRNAs can also be localized, we injected mRNAs of HrPOP-1, HrZF-1, HrPet-1, and HrPet-2 mRNAs. These mRNAs were also localized at the posterior pole (data not shown). We noted that embryos into which the type II mRNAs were injected had faint signals in blastomeres of the animal hemisphere (Fig. 1B). No such signal was observed in the type I mRNA-injected embryos (Fig. 1A). This is consistent with the finding that only a portion of the type II mRNA is localized to the posterior pole, and the remainder of it is uniformly distributed (Sasakura et al., 2000).

The specificity of localization of exogenous mRNAs suggests the correct localization. In order to confirm this, the localization of exogenous mRNAs in other stages was compared with that of the corresponding endogenous mRNAs. Type I exogenous mRNAs were detected at the cortex of unfertilized eggs (Fig. 1E) and were then localized at the posterior pole of two-cell embryos (Fig. 1F), suggesting that injected type I mRNAs bound to the cortex of unfertilized eggs and were then transported to the posterior pole. This localization pathway was identical to that of the endogenous mRNAs previously observed (Sasakura et al., 2000). Next, we tested the localization pathway of the injection of type II exogenous mRNAs. Endogenous type II mRNAs were distributed uniformly in unfertilized eggs, and the localized signal of type II mRNAs was rarely detected at the two-cell stage. In unfertilized eggs, the exogenous type II mRNAs did not accumulate in the cortex, in contrast to type I mRNAs (Fig. 1G), which is consistent with the localization pattern of the endogenous mRNAs. However, we detected posterior localization of type II exogenous mRNAs at the two-cell stage (Fig. 1H). This difference may be due to the different amount of exogenous mRNAs from endogenous ones. At least, because the localization pattern of type II mRNAs in the unfertilized eggs was truly different from that of type I mRNAs, the exogenous type II mRNAs may be carried to the posterior pole via a different localization mechanism from that of type I mRNAs. The localization pattern of the exogenous mRNAs appears to be the same as that of the endogenous mRNAs.

The 3' UTR Is Sufficient for the Posterior Localization

In the above section, we demonstrated that the system we used worked well for the study of the localization mechanism. Using this system, we tried to identify the localization elements of the postplasmic RNAs. Studies on maternally localized RNA in Drosophila and Xenopus eggs have revealed that the 3' UTRs are sufficient for the localization of many RNAs (reviewed in Mcklem, 1995; Bashirullah et al., 1998). Therefore, we considered it possible that the localization position was the same as that of the corresponding endogenous mRNAs (Sasakura et al., 1998a, 2000), suggesting that correct localization occurred via the normal localization pathway. Uninjected control embryos did not show any signals, indicating that the anti-digoxigenin antibody did not cross-react with unknown epitopes (Fig. 1D). To show that the localization of exogenous mRNAs is specific for mRNAs that are endogenously localized, Gfp and lacZ mRNAs were injected. Neither of these mRNAs was localized at all (Fig. 1C, and data not shown), suggesting that localization of exogenous mRNAs is specific to the nucleotide sequence of endogenously localized mRNAs. To test whether exogenous mRNAs of other type I or type II mRNAs can also be localized, we injected mRNAs of HrPOP-1, HrZF-1, HrPet-1, and HrPet-2 mRNAs. These mRNAs were also localized at the posterior pole (data not shown). We noted that embryos into which the type II mRNAs were injected had faint signals in blastomeres of the animal hemisphere (Fig. 1B). No such signal was observed in the type I mRNA-injected embryos (Fig. 1A). This is consistent with the finding that only a portion of the type II mRNA is localized to the posterior pole, and the remainder of it is uniformly distributed (Sasakura et al., 2000).

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FIG. 2. The 3' UTR is sufficient for localization at the posterior pole. (A) Illustration of the structures of injected mRNAs. Xenopus β-globin UTR is shown by a closed bar. (B) A HrWnt-5 5' UTR+ORF mRNA-injected embryo. The mRNA failed to be localized at the posterior pole. (C) A GFP ORF+HrWnt-5 3' UTR-injected embryo. A clear signal was observed at the posterior pole (arrowhead). (D) A HrPet-3 5' UTR+ORF mRNA-injected embryo. (E) A GFP ORF+HrPet-3 3' UTR-injected embryo. A signal was detected at the posterior pole (arrowhead). Scale bar, 100 μm.

FIG. 1. Localization of digoxigenin-labeled exogenous mRNA in ascidian embryos. Exogenous mRNA was detected with anti-digoxigenin AP Fab fragment (Roche). Posterior pole is toward the right for (A–D). (A) Localization of HrWnt-5 exogenous mRNA (arrowhead). (B) Localization of HrPet-3 exogenous mRNA (arrowhead). (C) GFP exogenous mRNA was not localized at the posterior pole. Blastomeres of the vegetal half had the weaker signal because of the yolk. The shade at the vegetal half looked as if there was a localized signal at the cortex, but actually, GFP mRNA was dispersed uniformly. (D) An un.injected control embryo did not show any signals. (A–D) Eight-cell-stage embryos, lateral view. (E) Exogenous HrWnt-5 mRNA was detected at the cortex of an unfertilized egg. (F) Exogenous HrWnt-5 mRNA was localized at the two-cell stage. (G) Exogenous HrPet-3 mRNA was not localized at the cortex in an unfertilized egg. (H) Exogenous HrPet-3 mRNA was localized posteriorly at the two-cell stage. Scale bar, 100 μm.
necessary for localization. Next, if the 3'ET fragment is the only fragment that has a set of localization elements, deletion of this fragment from the 3' UTR would abolish the localization ability. To test this, we deleted the 3'ET from the 3' UTR. The deletion of the 3'ET region completely abolished the localization activity (5'ET, 5'EV, and 5'S fragments in Fig. 3B), suggesting that other regions of 3' UTR did not have a complete set of localization elements. Although 3'ET alone can direct GFP mRNA to the posterior pole, the localization efficiency was a little weaker than that of the full-length 3' UTR, because the localized signal of GFP-3'ET mRNA was slightly but obviously weaker than that of full-length 3' UTR (data not shown). This may indicate that another region in the 3' UTR of HrWnt-5 mRNA enhances localization.

FIG. 3. Deletion of HrWnt-5 3' UTR and its effect on the posterior localization. (A) The structure of the GFP ORF+HrWnt-5 3' UTR construct. Arrows indicate the restriction sites (S, SacI; EV, EcoRV; ET, EcoT22I) (B) Diagram of deletion of 3' UTR using restriction enzymes, and the ability of the deleted mutant mRNA to localize. (C) Diagram of deletion of the 3'ET construct from either the 3' or 5' end, and the ability of the deleted mutant mRNAs to be localized. Gray ovals indicate the positions of UGREs. Numbers within parentheses are the % of embryos that showed strong localization signals of exogenous mRNAs. The WLS region is shown by a hatched bar.
localization. Despite this insufficiency, it is evident that a complete set of the localization element(s) of HrWnt-5 mRNA was located within the 3′ ET fragment, and that the other regions of the 3′ UTR were almost dispensable for the proper localization.

To map the minimal localization elements within the 3′ ET, we deleted the 3′ ET from either the 5′ or 3′ end. The results are shown in Fig. 3C. When about 80 nucleotides (nt) were deleted from the 3′ end of the 3′ ET (3′ ET/3′ d80), the localization was normal. However, deletion of an additional 20 or 40 nt (3′ ET/3′ d100 and 3′ ET/3′ d120) gradually lowered the localization activity. Therefore, one important localization element must exist around the border between these constructs. Deletion from the 5′ end revealed the existence of another minimal localization element. When 30 nt was deleted from the 5′ end, the 3′ ET/5′ d30 mRNA showed the normal localization. However, when 77 nt was deleted from the 5′ end of 3′ ET(3′ ET/5′ d77), the localization activity was severely decreased. In most embryos (about 85%), the mRNA did not localize, and in the remaining 15% of embryos, there was only a faint localization signal. Therefore, the 5′ end of 3′ ET has another indispensable localization element. These data suggest that there are at least two independent elements necessary for the localization of HrWnt-5 mRNA. We named the smallest sequence sufficient for the localization WLS (hrWnt-5 Localization Sequence; Fig. 3C). Elements critical for the localization must exist at both ends of WLS.

**Two UG Dinucleotide Repetitive Sequences Are Necessary for Posterior Localization of WLS**

From the results described above, we concluded that the minimal localization elements necessary for localization
exist within WLS. Furthermore, the results obtained by the deletion of 3'ET indicated that portions of the localization elements must exist near both ends of WLS. To identify these elements, we introduced nucleotide substitutions in several parts of WLS. If an essential localization element exists in a specific region of WLS, the disruption of the region will abolish or weaken the localization activity.

The results obtained from this experiment are shown in Fig. 4. The nucleotide substitutions introduced at region 1 (R1) and region 5 (R5) had large effects on the localization. Eighty-three percent of embryos into which R1-deficient WLS was injected did not show any localization signal, and the remaining 17% showed such faint signals at the posterior pole that we recognized the signal only in PBST buffer (after the treatment of embryos with BABB, the signals were lost). The disruption of R5 had an even stronger effect. None of the injected embryos showed any recognizable signal. In contrast to these two regions, nucleotide substitutions of other regions did not affect the localization at all, suggesting that these other regions are not necessary for the posterior localization. As shown in Fig. 4B, R1 and R5 are located near the 5'/H11032 end and 3'/H11032 end of WLS. Considering the results of a series of deletions of WLS, the effects of the nucleotide substitutions are convincing. Both results indicate the existence of two localization elements near the 5' and 3' ends of WLS. We compared the nucleotide sequences

FIG. 5. G residues within UGRE1 and UGRE2 are necessary for localization. (A) Nucleotide sequences of R1 and R3. UGREs are shown in italics. The As under the sequence indicate the guanine residues which were substituted by adenine. (B) Diagrams of mutation-containing mRNAs and their ability to be localized. The first and second yellow symbols indicate UGRE1 and UGRE2, respectively. The G and A in the yellow symbols indicate the normal (G) or mutated (A) UGRE. (C) The localization efficiency of 3'ET/3'd60/UaA mRNA. (a) Localization of 3'ET/3'd60 mRNA. This mRNA was localized correctly at the posterior pole (arrowheads). (b) Localization of 3'ET/3'd60/UaA mRNA. No localized signal was detected at the posterior pole (arrowheads).
of R1 and R5 and found that the regions contain highly characteristic motifs. Both R1 and R5 are rich in U and contain a UG dinucleotide repeat. R1 contains -UUGUUGUG-, and R5 contains -UUGUUGUGUG-. We named these two elements UGRE1 and UGRE2, for Uridine-Guanosine Repetitive Element.

There is no UGRE in the WLS sequence other than UGRE1 and UGRE2, suggesting the specificity of these elements. Surprisingly, R2 and R6, neither of which is necessary for localization, are also U-rich (18 U's/25 nucleotides for R1, 11/20 for R2, 14/20 for R5, and 13/20 for R6). If U-richness was the most important feature of WLS localization elements, stronger effects of the disruption of R2 and R6 than observed would be expected. However, the possibility that these two regions act redundantly still remains. To test this, we generated double mutations of R2/3 (positive control) and R2/6, and checked the localization activity of these mRNAs. These mRNAs showed almost the same (normal) localization activity, suggesting that R2 and R6 are completely unnecessary for the localization. Therefore, U-richness may not be the primary feature of the localization element. Rather, the UGRE structure, G residues flanked by Us, may be important. To confirm this possibility, we disrupted UGREs by replacing the Gs of UGRE1 and/or UGRE2 with As, and the effect on the localization was tested. Near UGRE1, another G residue, -uuuuGuuu-, existed, and in the nucleotide substitution experiment, this region was also disrupted along with UGRE1. Therefore, this G residue was also replaced with A (Fig. 5A). When the Gs of either UGRE1 or UGRE2 were replaced, the mRNAs showed almost the normal localization activity (Fig. 5B). In contrast, when the Gs of UGRE1 and UGRE2 were replaced simultaneously, the localization efficiency of the mRNA was extensively reduced (Figs. 5B and 5C). Only 27% of embryos showed a localization signal, and moreover, the localization signal was too weak to recognize in BABB solution. Therefore, the G residues in UGRE1 and UGRE2 play an important role in the WLS localization. This result also suggests the importance of the U-richness within and around the UGRE. This is because the nucleotide substitution of R1 (including UGRE1) or R5 (including UGRE2) was sufficient for the disruption of localization, while G-replacement of either UGRE1 or UGRE2 was not sufficient for the disruption of localization. From these results, we conclude that two UG dinucleotide repetitive sequences, UGREs, are the critical localization element necessary for the localization of WLS. Finally, to test whether R1/R5 was sufficient for the localization, two copies of R1 were tandemly conjugated with a green fluorescent protein reporter mRNA (2*R1), and the localization of this RNA construct was tested. 2*R1 did not have any localization activity (0%; n = 28), suggesting that R1 (including UGRE1) alone is not sufficient for localization. There seem to be several reasons why the tandem repeat of R1 does not suffice for the posterior localization, as discussed below.

The UGRE Is Conserved among the 3' UTRs of Type I Postplasmic mRNAs

The above results clearly showed that two UGREs are included within the localization elements of one of the type I mRNAs, HrWnt-5. The next question we asked is whether the UGRE is conserved throughout type I mRNAs. To answer this question, we mapped the localization element of another type I mRNA, HrPOPK-1.

The 3' UTR of HrPOPK-1 mRNA was deleted by restriction enzymes, and the localization activity of these mRNAs was checked. As shown in Fig. 6B, MB was the smallest fragment that had strong localization activity. MB is not located near the 3' end of the 3' UTR, unlike the case of WLS, suggesting that the position of the localization sequence is not restricted to a specific area (e.g., near the 3' end) of the 3' UTR. We then compared the primary structures of WLS and MB/3'd604. Although no sequence identical with UGRE1 and UGRE2 was found in MB/3'd604, there were several U-rich motifs (Fig. 6C). Especially, the second U-rich motif within MB/3'd604 consists of six repeats of UG, suggesting the conservation of UGREs within type I mRNAs. To further explore the conservation within type I mRNAs, we searched for UGREs in the 3' UTR of another type I mRNA, HrZF-1. We found two UGREs near the 3' end of the 3' UTR (Fig. 6D). One is UUGUUGUGUG, and the other is UUGUGUGUG. These UGREs are close to each other (the distance between these UGREs is only 227 nt long), implying the correlation of these elements to the localization of HrZF-1 mRNA. Although it remains necessary to test the function of these elements in localization, these data suggest the global conservation of UGREs within type I mRNAs and the likely importance of these elements for the posterior localization.

Identification of the Localization Element of HrPet-3 mRNA

The difference between the localization patterns of type I and type II mRNAs may be based on differences in their localization elements. Therefore, it is important to compare the localization elements of type I and type II mRNAs in order to understand how the different localization patterns are achieved by using the same cytoskeletal elements (Sasakura et al., 2000). To do that, we mapped the localization element of HrPet-3 mRNA. As shown in Figs. 7A and 7B, all of the mRNAs that included the 3'H region were localized effectively, whereas mRNAs that lacked 3'H failed to be localized. Therefore, all of the localization elements for HrPet-3 mRNA localization exist within the 3'H fragment. From the localization results for the deletion constructs of 3'H (Fig. 7C), we concluded that 3'H/3'd125 is the smallest fragment sufficient for the localization. We named 3'H/3'd125 PLS (hrPet-3 Localization Sequence).
When PLS was deleted from either the 3' or 5' end, the localization ability of PLS was diminished gradually (Fig. 7C). For example, when we deleted 20 or 40 nt from the 3' end of PLS, the localization ability decreased. A 60-nt deletion led to further loss of the localization ability, and when 80 nt was deleted, the RNA completely lost its ability to localize (Fig. 7C). This suggests that there may be several localization elements within PLS.

To identify these localization elements of HrPet-3 mRNA, we introduced a mutation into every 20 nt of PLS and checked the localization ability of these mutated mRNAs. If there is a core localization element that acts dominantly to specify the localization, the disruption of the area containing the element would reduce the localization ability.
efficiency. However, as shown in Fig. 8B, the localization ability of these mutated mRNAs was almost the same as that of PLS. Therefore, there is not a critical element in PLS, in contrast to WLS. Rather, these results suggest that PLS has several equivalent localization elements. These elements may act redundantly, and one mutation every 20 bases would not have worked. Some of the equivalent localization elements may be U-rich motifs (URMs), because (1) many URMs are observed within PLS and (2) some deletion mutants of PLS that lost a URM had decreased localization activity (3'H/3'd145, 185, 205, and 225 in Fig. 8A).

**DISCUSSION**

In this study, we identified new examples of maternal mRNAs whose localization is mediated by their 3' UTRs.
The localization signals of all of the maternal mRNAs examined thus far, except gurken mRNA, whose localization element is mapped to both the 5' and 3' UTR, are mapped to the 3' UTR (Micklem, 1995; Bashirullah et al., 1998; Saunders and Cohen, 1999; Thio et al., 2000). The conservation is interesting because the timing and position of mRNA localization vary among organisms. The function of 3' UTRs in mRNA localization is conserved in not only eggs but also somatic cells (Davis and Ish-Horowicz, 1991; Kislauskis et al., 1994; Bullock and Ish-Horowicz, 2001). Therefore, mRNA localization using the 3' UTR is a common strategy of all types of cells, including eggs and oocytes, and most maternal RNA localization is mediated by the 3' UTRs, regardless of the timing and position of localization. Yet, more intriguing variations in this theme lie beneath the general principle.

The localization element of HrWnt-5 mRNA was found to be composed of two UGREs. The UGRE appeared to be a

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**FIG. 8.** Mutations introduced into the HrPet-3 PLS fragment did not affect its localization. (A) Nucleotide sequence of HrPet-3 PLS fragment. The number of nucleotides is shown on the right. U-rich motifs are shown by boxes. Arrows indicate the deletion mutants of Fig. 7C. (B) Diagram of mutated mRNAs and their ability to be localized. Black boxes represent the positions of the mutations.
common localization element of type I mRNAs. We identified two features of UGRE. One is that the element is rich in Us, and the other is that it has several G residues. Many U-rich motifs (URMs) are also observed in PLS (the localization sequence of one of the type II mRNAs, HrPet-3), and some URMs appeared to function in HrPet-3 mRNA localization. This common feature is surprising because the localization of type I mRNAs and type II mRNAs is different (Sasakura et al., 2000). It is possible that U-rich sequences are used as a common framework of the localization elements in both type I and type II mRNAs, and some other residues embedded in the U-rich frame create different localization patterns. The idea is supported by the fact that the 3' UTRs of ascidians are very U-rich and it is true of both type I and type II mRNAs (35% for HrWnt-5 and HrPOPK-1; 36% for HrZF-1; 37% for HrPet-1; 39% for HrPet-2 and HrPet-3). If U-richness was the only feature of the localization elements of type I mRNAs, then the different localization patterns of type I mRNAs and type II mRNAs could not be explained. For type I mRNAs, G-richness is necessary for acquisition of the difference. In addition, the fact that two UGREs are close to each other may be important, because the necessity for two elements may serve for the more stringent selection of type I mRNAs from other maternal mRNAs. Not only HrWnt-5 WLS, but also the HrZF-1 3' UTR, has two UGREs, in accord with this hypothesis. The localization sequence of HrPOPK-1 mRNA has only one UGRE. However, it contains some UGRE-like motifs (Fig. 6C), and one of these elements may act for localization together with UGRE. A U-rich localization element was also reported in the case of Vg1 (VM1, UUUCUA; Gautreau et al., 1997). The cross-species conservation of U-rich sequences as the localization elements of mRNAs is interesting. It might be possible to inject the Vg1 mRNA into the ascidian eggs and assess whether this mRNA shows any localization in the heterologous system.

Although two UGREs are the indispensable localization element of HrWnt-5 mRNA and are highly conserved among type I mRNAs, the insufficiency of UGREs for the localization is suggested by the fact that a tandem repeat of R1 did not direct GFP mRNA to the posterior pole. Our preliminary data, which show that neither a tandem repeat of R1-R5 nor mutated WLS in which the sequence between R1 and R5 was replaced by the lacZ sequence has localization ability, support the insufficiency of UGRE. There are at least two possible explanations for these results. One is that there is another critical localization element between two UGREs. Although there is not a characteristic motif other than R2 and R6 in the area, this possibility still remains. The other possibility is that the UGRE forms a secondary structure with some other region(s). The secondary structure of WLS was investigated, but we could not find any specific motif. However, to predict the secondary structure of an mRNA in vivo is difficult, and it is possible that UGREs form some structures like hairpin-loops, and the localization machinery recognizes these structures.

One important question is in which step(s) UGRE is involved in the localization of type I mRNA. As previously reported, type I mRNAs are localized to the posterior pole in two phases (Sasakura et al., 2000). Type I mRNAs localized at the cortex of unfertilized eggs move to the vegetal pole during the first phase of ooplasmic segregation after fertilization; then, they are transported to the posterior pole during the second phase of ooplasmic segregation. Therefore, there are three steps of localization in which UGRE is involved. One is binding to the cortex of unfertilized eggs, another is accumulation at the vegetal pole, and the other is transport to the posterior pole. The first two steps are closely related, because both of them are dependent on actin filaments, whereas the third step is dependent on microtubules (Sasakura et al., 2000). We favor the idea that UGRE is needed for the first two steps, binding of mRNAs to the cortex and/or accumulation at the vegetal pole. This is because we did not observe UGRE-disrupted mRNA (3'E/T/3'd60/UaA of Fig. 5) remaining at the vegetal pole of embryos. Rather, UGRE-disrupted mRNA seemed to be distributed throughout the cytoplasm. If our idea is right, it is of interest whether UGRE is also needed for the last step of RNA localization (transport to the posterior pole by microtubules) or another distinct element independently determines this step.

Considering the predominant effect of UGRE on RNA localization, some RNA-binding proteins must bind to UGRE. What RNA-binding protein(s) might bind to UGRE? There are two known RNA-binding proteins whose specific binding sequences are similar to UGRE. One of them is Musashi (Msi). It was shown by SELEX analysis that the consensus binding sequence of Msi is GU3-(G/A)G (Okabe et al., 2001). This sequence is very similar to the localization element we identified in this study. Ascidian msi homologs were isolated from Halocynthia and Ciona (Kawashima et al., 2000), and the mRNAs encoding them are maternally expressed. Therefore, it is possible that Msi protein is expressed in eggs and regulates the posterior localization of mRNAs. The other RNA binding protein is the Tristetraprolin zinc finger protein. This protein has C3H-type zinc fingers and binds to an AU-rich sequence of TNF-α (Carballo et al., 1998). The purine-interrupted U-rich feature is similar to the UGRE. Moreover, maternal C3H-type zinc finger proteins were isolated from Caenorhabditis elegans. One of them, PIE-1 protein, was shown to act as an RNA-binding protein (Teneinhaus et al., 2001). Therefore, C3H-type zinc finger proteins may be generally used as RNA-binding proteins. One of the type I mRNAs, HrZF-1, encodes a C3H-type zinc finger protein (Sasakura et al., 2000), and it is possible that HrZF-1 protein is one of the ligands of UGRE of type I postplasmic mRNAs. This would essentially be a type of classical autogenous regulation as applied to mRNA localization.

In contrast to HrWnt-5 mRNA, the minimal localization element of HrPet-3 mRNA was not identified. The results of the deletions and nucleotide substitutions of PLS suggest the existence of several localization elements that act
redundantly in the PLS region. It also remains possible that, although there is a central localization element in PLS, our nucleotide substitution experiment did not disrupt the element completely (for example, a 20-base substitution would disrupt half of the element), resulting in too slight an effect on localization to make a detectable difference from controls. However, we favor the former explanation for two reasons. One is that even a partial disruption of a central element may conceivably change the localization. The other and more compelling reason is that the deletion experiment of PLS clearly indicated the existence of several localization elements, including some URMs (Fig. 8A). Although URMs seem to be important for the localization of PLS, no UGREs are observed in PLS, suggesting the difference between PLS and WLS. URMs are not the only localization elements of PLS, because a 56-nt deletion from the 5' end of PLS (3' UTR of d85/5' d56) lessened the localization activity although there is no URM within the deleted region. To demonstrate the redundancy of localization elements, additional experiments, such as the introduction of mutations in two regions at a time, will be necessary. In addition, it is necessary to determine whether the redundant element is common in the type II mRNAs. It is also of interest to know whether the two phases of the localization of type II mRNAs (described in Introduction and Results) are dependent on the same or different localization element(s). The localization timing and mechanisms of the two phases are clearly distinct from each other. However, our previous study (Sasakura et al., 2000) revealed that the disruption of the first phase of localization (during the ooplasmic segregation) by nocodazole also affected the localization pattern of mRNAs at the second phase (during the second cleavage). Therefore, either of the two ideas remains possible: (1) the two localization pathways are dependent on different localization element(s) or (2) they are caused by the same localization element(s). Considering the difference of the two localization events, the former possibility seems more likely based on the presently available information. However, if the latter is the case, it will be of interest whether the two localization events are sequential ones using the same factor(s) and cytoskeletal elements, though the timing and mechanisms seem different.

The study of localization elements of maternal mRNAs was started in Drosophila and Xenopus (Yisraeli and Melton, 1988; MacDonald and Stroehl, 1988). For many mRNAs, a partial 3' UTR sequence was shown to be sufficient for the localization (summarized in Bashirullah et al., 1998). However, identification of a minimal localization element has not yet been achieved for most mRNAs, except bicoid, K10, and Vg1 (Mowry and Melton, 1992; MacDonald et al., 1993; Serano and Cohen, 1995; Deshler et al., 1997; Gautreau et al., 1997). For the bicoid and K10 mRNAs of Drosophila, the importance of secondary structure was suggested. In contrast, the primary structure of repetitive elements seemed important for Vg1 mRNA localization (Deshler et al., 1998; Harvin et al., 1998). Presently, it cannot be determined which mechanism is more general. For such a global understanding of the mechanism of localization of mRNAs, identification of the minimal localization element of many other mRNAs of diverse organisms and their stages of development will be necessary. For such studies, ascidian mRNAs that show clear localization at the posterior pole are useful models, as our present study conveys.

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Localization Element of Ascidian Localized mRNAs


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