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In vivo RNA localization of *I factor*, a non-LTR retrotransposon, requires a *cis*-acting signal in ORF2 and ORF1 protein

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ABSTRACT

According to the current model of non-LTR retrotransposon (NLR) mobilization, co-expression of the RNA transposition intermediate, and the proteins it encodes (ORF1p and ORF2p), is a requisite for the formation of cytoplasmic ribonucleoprotein complexes which contain necessary elements to complete a retrotransposition cycle later in the nucleus. To understand these early processes of NLR mobilization, here we analyzed *in vivo* the protein and RNA expression patterns of the *I factor*, a model NLR in *Drosophila*. We show that ORF1p and *I factor* RNA, specifically produced during transposition, are co-expressed and tightly co-localize with a specific pattern (Loc+) exclusively in the cytoplasm of germ cells permissive for retrotransposition. Using an ORF2 mutated *I factor*, we show that ORF2p plays no role in the Loc+ patterning. With deletion derivatives of an *I factor* we define an RNA localization signal required to display the Loc+ pattern. Finally, by complementation experiments we show that ORF1p is necessary for the efficient localization of *I factor* RNA. Our data suggest that ORF1p is involved in proper folding and stabilization of *I factor* RNA for efficient targeting, through Loc+ patterning, to the nuclear neighborhood where downstream steps of the retrotransposition process occur.

INTRODUCTION

Non-LTR retrotransposons (NLRs) [retrotransposons lacking long terminal repeats (LTRs)], are a class of transposable elements that are mobilized by reverse transcription of an RNA intermediate (1). Multiple copies of NLRs inhabit variable fractions of the genome of most eukaryotes. Whereas most copies are defective, some are functional and their retrotransposition can result in several types of genetic effects including mutations associated with diseases in humans and mice (2). The *Drosophila* NLR *I factor* and the mammalian NLR L1 (3) provide well-known examples of the impact of NLR mobilization.

Most NLRs contain two open reading frames, ORF1 and ORF2, which are translated from a bicistronic messenger RNA that presumably also serves as a template for retrotransposition (4–6). The sequence of the ORF2 product (ORF2p) is relatively well conserved among NLRs. ORF2p is involved in reverse transcription and integration, and its endonuclease and reverse-transcriptase activities have been demonstrated *in vitro* for different elements (7–12). The sequence of the ORF1 product (ORF1p) is, in contrast, poorly conserved in evolution. ORF1p of human and mouse L1 are expressed in some carcinoma cells and can be isolated as a 40 kDa full-size protein (p40) which forms, in association with L1 RNA, high molecular weight cytoplasmic ribonucleoprotein (RNP) complexes (13,14). *I factor* ORF1p, expressed in bacteria or insect cells, shows both DNA and RNA binding properties and accelerates the annealing of complementary single-strand oligonucleotides without sequence specificity (15), similar to mouse L1 ORF1p when expressed in those heterologous systems (16–18). These *in vitro* experiments suggested that ORF1p from NLRs could act as a chaperone.

It is now known that the integrity of both ORF1 and ORF2 is required for retrotransposition (19–21), but our knowledge is limited on how and when the products of these ORFs interact with the RNA transposition intermediate during the mobilization process. The model NLR *I factor* allows the study of retrotransposition *in vivo*, making possible the tracking of specific

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transposition products in *Drosophila*. *I factor* transposes specifically in the oocytes of females (called SF) issued from mating males carrying active *I factor*s (inducer or I strains) with permissive females lacking active *I factor*s (reactive or R strains). A proportion of the eggs laid by the SF females fail to hatch. The severity of this sterility correlates with the frequency of transposition of *I factor*s [reviewed in (22)].

We have previously described the localization pattern of ORF1p produced by active *I factors* in the germ cells of SF females, showing that it is correlated to the element retrotransposition (23). We show here that *I factor* RNA and ORF1p generated by active *I factor*s during mobilization co-localize with a specific cytoplasmic pattern (Loc+), exclusively in permissive oocytes. By analyzing the expression patterns of mutated/deleted *I factor*s in transgenic females, we show that ORF2p is not involved in the Loc+ patterning and that localization of ORF1p/RNA requires an RNA *cis*-acting signal located in the sequence of ORF2. Finally, the expression and complementation analyses of an element deleted in ORF1 indicate that ORF1p is required (either in *cis* or in *trans*) for efficient Loc+ patterning of the RNA. This result suggests that some chaperone-like properties of ORF1p described *in vitro* (15,17), may apply *in vivo* to force proper folding of *I factor* RNA and enhance its targeting to the nuclear vicinity, favoring the efficiency of downstream steps of the retrotransposition process.

MATERIALS AND METHODS

Fly stocks

The JA (*white* and *yellow*) stock is a reactive (R) strain, permissive for *I factor* transposition. The strain w_{1118} is a standard inducer (I) stock. The transgenic lines HT1 and HT2 carrying several copies of the marked I-HAO1 element (23) were also used as I stocks. Stocks HH16 and Is37 are transgenic lines for constructs *hs*ORF1 and I-ΔAsu, respectively, and were established after P-element mediated transformation of the reactive strains *w*K and Cha (24). All strains are M in the P–M system of hybrid dysgenesis, thus transposition events cannot result from P-element activity.

Plasmid constructs

All constructs with internal deletions of the ORF2 region were derived from the element I-HAO1, a functional I element marked with the HA tag at the beginning of ORF1 in construct pCaSpeR/I-HAO1 (23), by performing restriction digests according to the sites indicated in Figure 2. Nucleotide positions and the corresponding sequence corrections were described previously (25,26). The integrity of the junction points was checked by sequencing. To obtain construct A, we cloned the thymidine kinase terminator, contained within an SmaI–EcoRI fragment from plasmid pBTK2, at the end of ORF1 in construct pCaSpeR/I-HAO1 cut with HpaI–EcoRI (Figure 2). To obtain construct pCaspeR/I-HAO1-fsO2, an 8 nt oligomer was ligated to EcoRV-digested pCAspeR/IHAO1. The I-ΔAsu construct contains an in-frame AsuII deletion within ORF1 and was derived from pI954, a plasmid containing a complete *I factor* cloned into vector pUC*hsneo*. Construct *hs*ORF1 was described previously (24). To obtain construct DF313/loc, a *lacZ*/*I factor* fusion, we generated the 552 nt localization fragment by PCR using primers 5′-GTATCTAGAACTTAGCTCAGCAC-sense and 5′-GACTAGTGGCTTGATGTATGCGG-antisense, digested it with XbaI/SpeI and cloned it at the 3′ end of a β-*gal* gene in plasmid DF313 cut with SpeI. In plasmid DF313, the promoter of the maternal alpha4-tubulin gene 67C drives the expression of the β-*gal* gene (D. Ferrandon, personal communication).

Transformations

All constructs derived from I-HA-O1 were cloned into the pCaSpeR4 transformation vector containing the mini-*white*+ gene as a selection marker (27). They were introduced into reactive JA flies (*y*,*w*) by P-element mediated transformation (28). Transposase activity was provided by the PUC*hs*Δ2-3 helper plasmid (Flybase ID: FBmc-0000938). Several independent transformed lines were generated and three were analyzed for each construct, with the exception of CI21, for which only one line showing a full expression of the transgene was obtained.

Complementation experiments

We performed reciprocal crosses between flies from stocks HH16 and Is37. The F1 female progeny underwent two heat-shock treatments of 1 h at 37°C, 1 day before and 1 h before dissection of the flies. The expression of I-ΔAsu transcripts in the ovaries of Is37/HH16 hybrids (with and without heat treatment) was specifically detected using a probe corresponding to *I factor* 5′UTR, absent in the *hs*ORF1 construct.

PCR detection of transposed copies

The method for obtaining flies in which the presence of transposed copies was investigated and the method for extracting their DNA was described previously (23). Briefly, the transgenic element integrated in w flies is linked to the w+ selection marker, while transposed copies are not. Therefore, after allowing a single cycle of transposition in heterozygote transgenic females, we selected w progeny and used PCR to detect transposed copies of the marked element in their DNA. The primers used were 5′-TTACCATACGACGTCCCAGA (sense) which overlaps the HA tag and 5′-GATCAGATCTGATCCTTTTAGA (antisense) which is specific to the frameshift mutation introduced in ORF2. The size of the expected product was 1354 nt.

In situ hybridization

Ovaries were dissected in ice cold phosphate-buffered saline and processed for *in situ* hybridization as described previously (29). The probe consisted of a PCR amplification product of *I factor* 5′UTR purified on a Qiagen column and labeled with digoxygenin using the Boehringer nick-translation kit. Occasionally, we used a similarly prepared probe representing a short segment of ORF1 or ORF2.

Immunofluorescence

Antibody staining was performed as described previously (23). The antibodies used were mouse anti-HA12CA5 (1:500 dilution) (Roche) and FITC-conjugated anti-mouse IgG (1:200 dilution) (Vector) or rat anti-HA high affinity 3F10 (1:500 dilution) (Roche) and FITC-conjugated anti-rat antibodies (1:500 dilution) (Vector).

RESULTS

ORF1p and the *I factor* transcript co-localize temporally and spatially during oogenesis

We have analyzed the expression pattern of the transcripts produced by active *I factor*s during retrotransposition and compared it with the pattern previously identified for ORF1p [(23), and Figure 1a, panels B and D]. Briefly, ORF1p produced in nurse cells is transported to the oocyte cytoplasm from the early stages of oogenesis, where it accumulates at the posterior pole until mid-oogenesis. At this stage ORF1p re-localizes to the oocyte anterior pole.

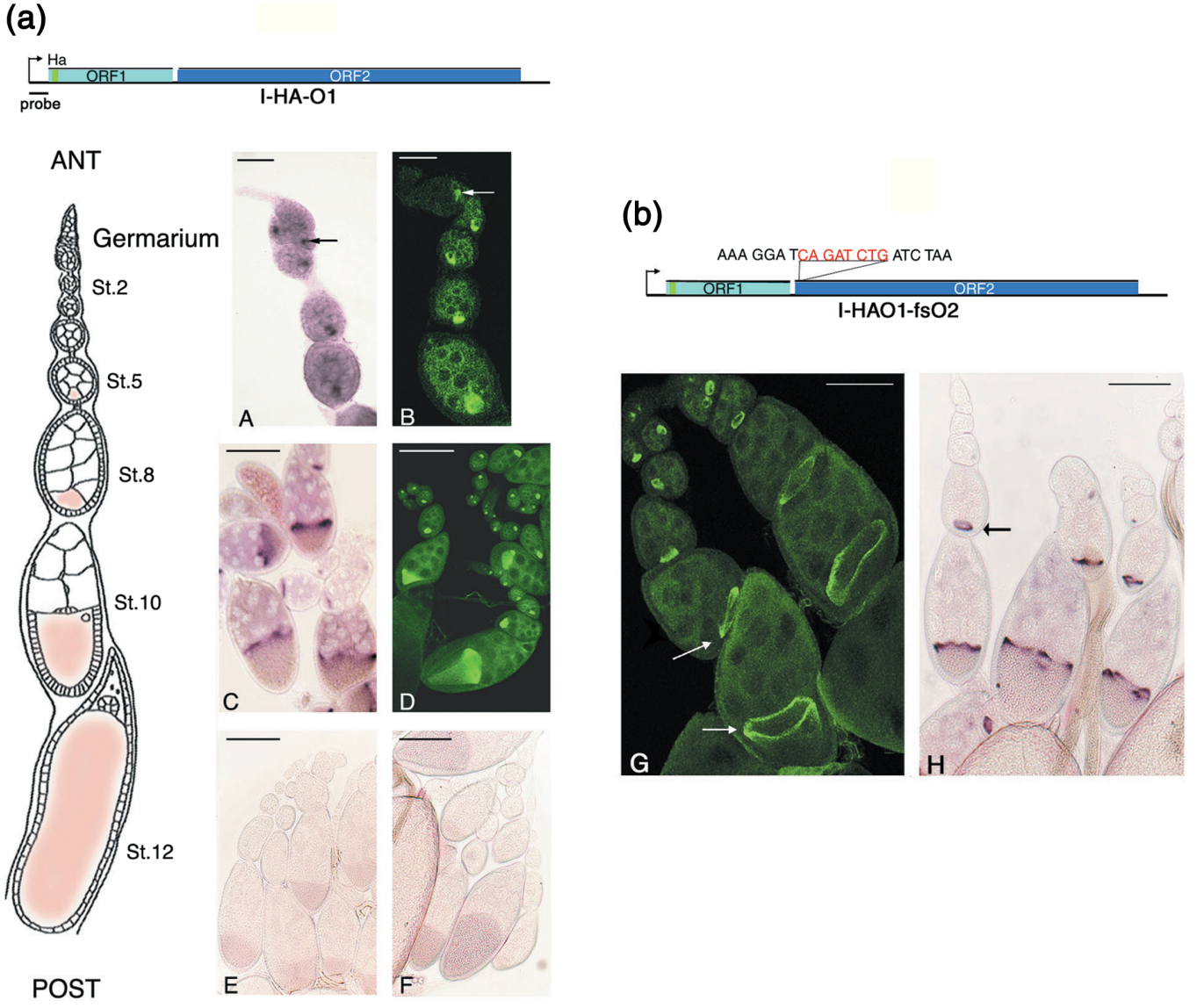

Figure 1. The Loc+ phenotype. (**a**) The top diagram represents I-HA-O1, a functional *I factor* carrying the HA tag at the N-terminus of ORF1p (23). The arrow indicates the transcription start site of the *I factor* driven by an internal promoter contained within its 5′UTR. The black bar underneath indicates the sequence used as the probe in *in situ* hybridizations. Below, the left panel shows a schematic diagram of an ovariole (the developmental unit of a *Drosophila* ovary where oogenesis takes place). At the anterior tip of the ovariole (ANT), in the germarium, stem germ cells divide to produce 16 cell cysts; one of these cells becomes the oocyte and the other 15 become nurse cells. Each cyst, surrounded by a layer of somatic follicle cells, constitutes an egg chamber. Egg chambers grow and mature as they progress from the germarium to the posterior part of the ovariole (POST), which ends in the oviduct. The different stages of maturation can be identified from the relative volume of the oocyte (32). (A, C, E, F and H) *In situ* hybridizations designed to detect *I factor* transcripts on whole-mount ovaries. (B, D and G) Immunostaining for the detection of HA-tagged ORF1p. (A–D) The Loc+ phenotype can be observed in the ovaries of SF females generated by crossing I males carrying the active tagged I-HA-O1 element with R females. *I factor* transcripts (A) and ORF1p (B) accumulate in the cytoplasm of the oocytes from the very early stages of oogenesis, as soon as the pro-oocyte is determined in the germarium (arrows). At mid-oogenesis (stages 8 and 9, and thereafter), when the oocyte nucleus migrates from the posterior to the antero-dorsal pole of the cell, both the *I factor* RNA (C) and ORF1p (D) are also re-localized and concentrate at the anterior cortex of the oocyte. *I factor* transcripts are not detected in the ovaries of R flies, which are devoid of active *I factor*s (E), nor in the ovaries of I flies, where active *I factor*s are silenced (F). (**b**) Loc+ pattern of I-HAO1-fsO2. The insertion of 8 nt, in red in the diagram, in I-HAO1 near the 5′ end of ORF2 creates a frameshift in ORF2 and abolishes the transposing capacity of this element. The dynamic expression pattern of ORF1p (G) and RNA (H) is unchanged, indicating that the localization process does not require the participation of the ORF2 protein. [The concentration of ORF1p at the periphery of the oocyte anterior poles appears like green crescents in the oblique views of stages 8 and 9 egg chambers in (G) (arrows)]. Scale bars in (A) and (B) are 10 μm and in C–H are 50 μm.

This movement coincides temporally and spatially with the change of microtubule polarity and the migration of the nucleus from the posterior to the anterior-dorsal pole (30,31). In late developmental stages, coincident with nuclear membrane disassembly and resumption of meiosis I, ORF1p is no longer detected in the oocyte. We synthesized probes representing the *I factor* 5′UTR and performed *in situ* hybridization on the ovaries of SF females resulting from crosses between R females and I males containing several active copies of the *I factor* (either from natural I strains or from transgenic strains containing the functional tagged I-HA-O1 element). As controls, we tested the ovaries of R and I females (Figure 1a, panels E and F). We detected a strong expression of *I factor* RNA exclusively in SF females (Figure 1a, panels A and C). This expression pattern was identical to that of ORF1p, i.e. accumulation in the cytoplasm of early oocytes, relocalization from the posterior to the anterior pole at mid-oogenesis and extinction of the signal after stage 10b (Figure 1a, panels A–D). The co-localization of ORF1p and *I factor* transcripts during oogenesis strongly suggests that both molecular species interact and form RNP complexes during retrotransposition. We defined this dynamic subcellular localization pattern of ORF1p and RNA as the 'Loc+' phenotype.

To investigate how the different components of the *I factor* (ORF1p, ORF2p and RNA) interact and localize, we generated a frameshift mutation in ORF2 and several deletions downstream of the ORF1 sequence of the I-HA-O1 element (Figure 2). Using standard procedures, we introduced the mutated elements in the JA reactive strain and established several independent homozygous transgenic lines for each construct. In the following paragraphs we describe the analysis of the expression patterns of ORF1p (by histo-immunofluorescence) and of the transcripts (by *in situ* hybridization) produced by these *I factor*s in a standard permissive background; i.e. in females resulting from crosses of homozygous transgenic males to JA reactive females. We tested several independent transgenic lines per mutant (see Materials and Methods).

The ORF2 protein is not required for the Loc+ phenotype

We disabled the translation capacity of ORF2 from its first AUG codon by creating a frameshift mutation near its 5′ end (element I-HAO1-fsO2) (Figure 1b, panels G and H). Interestingly, for each independent line analyzed, ORF1p and the transcripts presented a Loc+ phenotype. We verified that the frameshift in ORF2 actually abolished the transposition capacity of the element I-HAO1-fsO2. For this, we adapted a PCR-based method (23) to detect specifically the HA tag from transposed copies in the male progeny of SF females

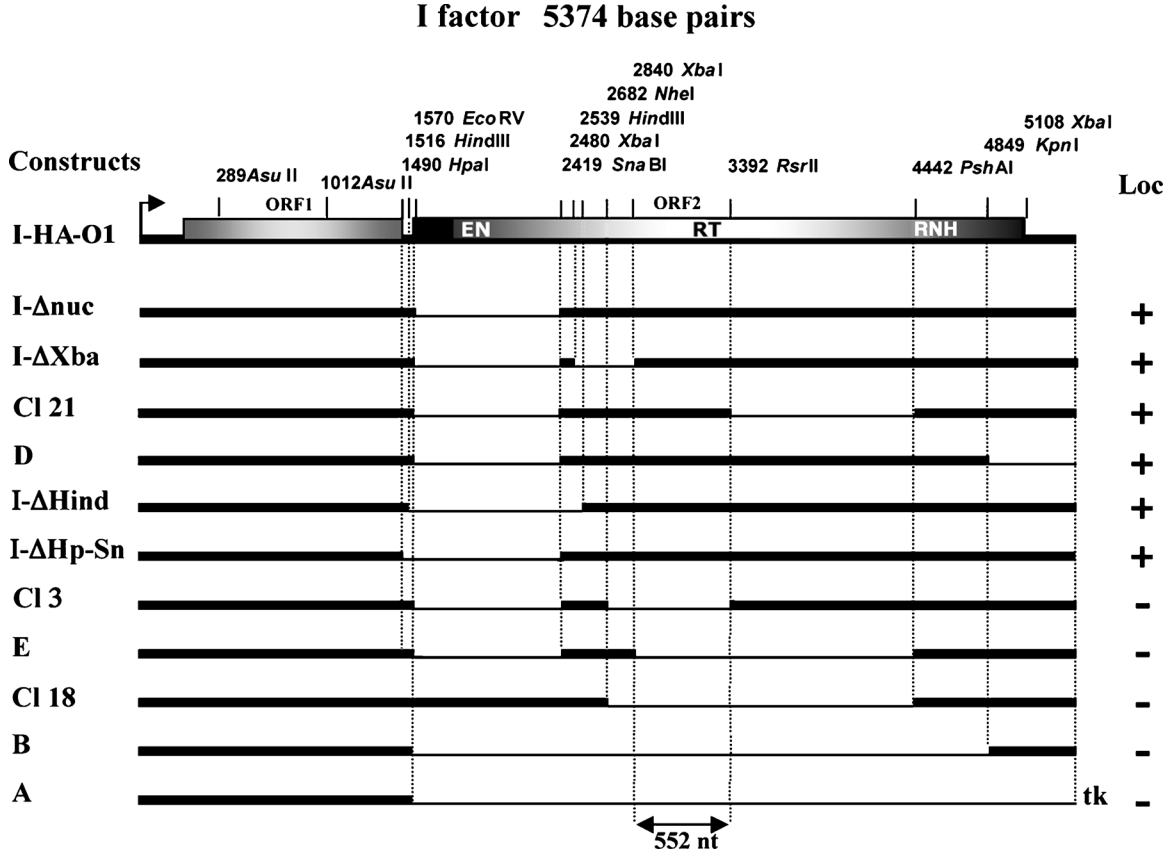

Figure 2. Schematic representation of the I-HA-O1 element and of its deletion derivatives. The arrow at the beginning of the 5′UTR indicates the transcriptional start site of the element. The relative locations of the endonuclease (EN), reverse transcriptase (RT) and RNAse H (RNH) domains within ORF2 are indicated. Restriction enzymes used to obtain the different constructs and their sites within the *I factor* sequence are shown. Thin lines represent the deleted sequences in each construct. The localization phenotypes with regard to ORF1p and the RNA are indicated at the right of each construct (Loc). tk represents the thymidine kinase terminator added to the 3′ end of construct A. The 552 nt stretch missing in all constructs displaying a Loc− phenotype is indicated.

(see Materials and Methods). Whereas 20–70% of the progeny contained transposed copies of the parental I-HA-O1 element, no transposition events were detected in a sample of 300 flies pooled from three independent transgenic lines carrying the I-HAO1-fsO2 element (100 flies/line). These results indicate that: (i) I-HAO1-fsO2 is unable to transpose at a detectable frequency; (ii) functional ORF2 products were not produced from AUG codons located downstream of the first AUG of ORF2, identified as the initiation codon of this ORF (5); (iii) in the absence of ORF2p, *I factor* RNA and ORF1p are transported and co-localize in oocytes as efficiently as when they are produced by transposing elements.

The Loc+ phenotype requires *cis*-acting sequences within ORF2

We examined the expression pattern of a construct B (see Figure 2), containing only the 5′UTR, ORF1 and the 539 nt of the 3′end of the element, which includes a putative nucleic acid binding domain at the end of ORF2 (26), the 3′UTR and four TAA repeats. To assess the effect of the 3′end region in the localization process, we replaced it by the herpes thymidine kinase terminator (*tk*) in construct A (Figure 2). For both the constructs and the three independent transgenic lines analyzed per construct, ORF1p was detected in the cytoplasm of nurse cells and oocytes. However, the intensity of the signals was weaker than that observed in females exhibiting a Loc+ phenotype. This signal was detected only at early stages of oogenesis (never later than stages 5–8). Interestingly, at stage 8, the weak fluorescent signal appeared spread throughout the cytoplasm of some oocytes, and was never accumulated at the anterior cortex of the cell. The *in situ* hybridization signal produced by the transcripts was also weak and diffuse throughout the cytoplasm of the nurse cells. This type of localization-defective phenotype will be referred to as a 'Loc−' phenotype (Figure 3A, B, and D). These results indicate that ORF1p and *I factor* sequences present in construct B were not sufficient to promote the Loc+ phenotype. Therefore, we analyzed several other deletion mutants, downstream of ORF1 to determine which sequences are required in *cis* for the patterning process (Figure 2). According to the expression patterns observed for ORF1p and the I transcripts, we defined two categories of mutants: (I) those displaying a Loc+ phenotype (Figures 3H–K and 4E), and (II) those displaying a Loc− phenotype similar to that presented by elements A and B (Figures 3C, E, F, and G and 4C). We did not observe another category of mutants that would exhibit a Loc+ phenotype for the transcripts and Loc− for ORF1p (or the reverse). Sequence comparisons of the diverse constructs and their corresponding phenotypes revealed that a 552 nt segment, located in a central region of ORF2, is absent in all the mutants exhibiting a Loc−phenotype (Figure 2).

The 552 nt segment drives the localization of a heterologous RNA

To further assess the significance of the *cis*-acting sequence identified above in the localization of the *I factor* RNA, we tested its capacity to localize a heterologous RNA expressed in the female germ line. We added the 552 nt fragment at the 3′ end of a construct (DF313) containing the bacterial *lacZ* gene under the control of the promoter of the maternal

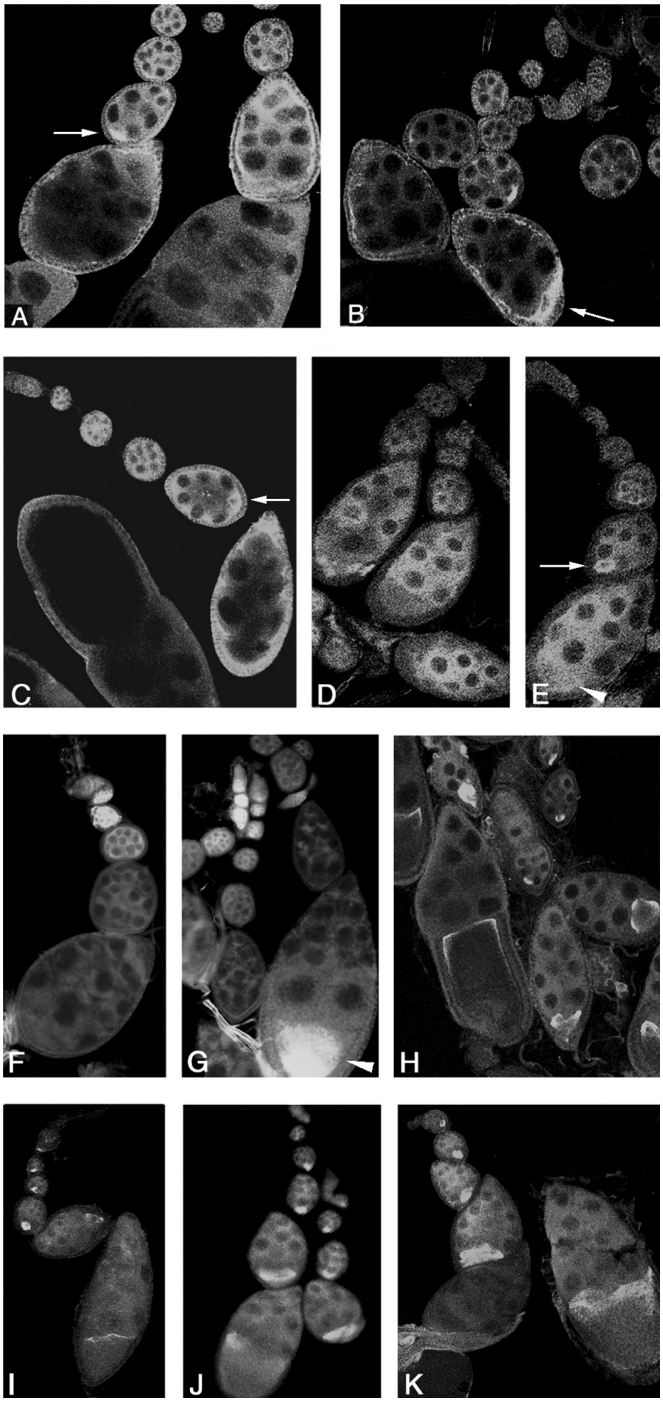

Figure 3. ORF1p expression produced by different *I factor* deletion derivatives. Immunostaining on ovaries of flies generated by crossing R JA females with transgenic males carrying either construct A (**A** and **B**), construct E (**C**), construct B (**D**), construct CI18 (**E**); construct CI3 (**F** and **G**), construct I-Δnuc (**H**), construct D (**I**), construct CI21 (**J**) or construct I-ΔHind (**K**). The constant and regular ORF1p expression pattern of Loc+ mutants shown in (H–K) complies exactly with the localization features of full-size elements (see Figure 1). In contrast, Loc− mutants (A–G) present a large variability in the ORF1p expression patterns within each transgenic line. (A and B) and (F and G) illustrate this variability in the same genetic background. However, two features are constant: ORF1p is not preferentially accumulated in oocytes at the early stages of oogenesis but may occasionally label the oocytes in stage 6 and 7 cysts (arrows in A, C and E). ORF1p is generally missing in stages 8 and 9 oocytes, but when present, it appears scattered all over that cell (arrowheads in E and G) and does not migrate to the anterior pole.

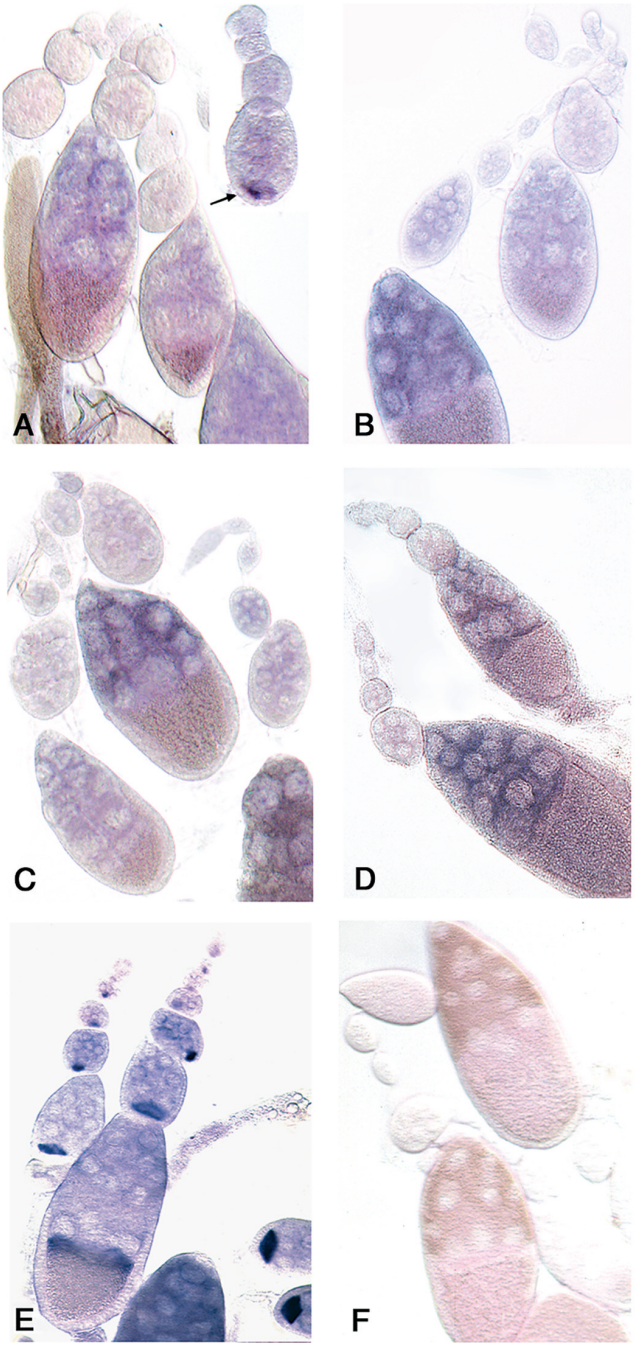

Figure 4. RNA expression pattern produced by different *I factor* deletion derivatives. *I factor* transcripts are detected as a blue precipitate following *in situ* hybridization on ovaries (A–E) (see Materials and Methods). The probe used does not reveal any *I factor* RNA in control R JA flies (**F**). Transcripts of all Loc− mutants appear scattered in all the 16 cells of young cysts and are accumulated in nurse cells after stage 10 as shown in (A–D). Exceptionally, the RNA appears accumulated in some oocytes at stages 6 and 7 (arrow in A), like ORF1p in some Loc− phenotypes (arrows in Figure 3A and C). In contrast, the phenotype of Loc+ mutants (E) is regular and identical to that of the full-size element. Construct A (**A**); construct B (**B**); construct CI18 (**C**); construct E (**D**) or construct I-Δnuc (**E**).

alpha4-tubulin gene 67C that drives the expression in oocytes. The chimera *lacZ/I factor* construct was called DF313/loc. We transformed reactive flies either with DF313/loc, creating transgenic lines 313/loc, or with the unmodified DF313 construct, creating transgenic lines 313. In control lines 313, *lacZ* transcripts were not transported to the anterior pole of oocytes (Figure 5B). In contrast, the chimera *lacZ*/loc transcripts localized to the oocyte anterior pole in lines 313/loc, exactly like *I factor* transcripts (Figure 5A). This suggests that the 552 nt fragment of ORF2 contains all the necessary sequences to interact with cellular motor proteins and drive the localization of *I factor* products.

A deletion in *I factor* ORF1 prevents an efficient expression of the Loc+ phenotype

To determine whether other *cis*-acting sequences contribute to *I factor* RNA localization, we analyzed the expression of the element I-ΔAsu (24), which lacked 723 nt between the two AsuII restriction sites of ORF1. I-ΔAsu produces a half size ORF1p, in which the zinc-knuckle region is removed (Figure 6). Males of the Is37 transgenic line homozygous for the I-ΔAsu element were crossed with JA females, and we analyzed the expression pattern of the transcripts and of the mutated ORF1 protein in the ovaries of their F1 progeny. This truncated protein appeared accumulated in the nurse cell cytoplasm. It appeared scarcely in oocytes and was not localized to the anterior pole (data not shown). The distribution of the transcripts was similar but, exceptionally, some very faint figures of localization at the anterior pole of stage 9 oocytes could be observed (Figure 6A, arrows). These observations suggest that in the *I factor* context, the 552 nt region within ORF2 is not sufficient to promote a precise distribution of *I factor* RNAs, and that sequences of ORF1 missing in I-ΔAsu and/or a functional ORF1p are necessary for their correct and efficient localization.

ORF1p can act in *trans* to restore the Loc+ phenotype

To test whether ORF1p and/or other *cis*-acting sequences in ORF1 are required for the localization of *I factor* RNA, we conducted complementation experiments introducing I-ΔAsu and full-length ORF1p into the same oocyte. For this, we performed reciprocal crosses between line Is37, containing the I-ΔAsu element and a line containing the construct *hs*ORF1 (HH16 line) (24). In the construct *hs*ORF1 the expression of ORF1p is driven by the heat-shock promoter *hsp*70. An abundant and ubiquitous expression of ORF1p can be induced in line HH16 by heat treatment at 37°C (data not shown). A control heat shock on Is37 females had no effect on the localization of the I-ΔAsu RNA (Figure 6B). In contrast, when Is37/HH16 females were subjected to heat treatment, a large proportion of I-ΔAsu transcripts was localized in the oocytes with a full Loc+ phenotype, exactly like complete *I factor* transcripts (Figure 6C). Such hybrid females also expressed the typical sterility syndrome correlated with *I factor* retrotransposition. These results indicate that (i) in addition to the localization signal in ORF2, ORF1p is required to promote an efficient localization of *I factor* RNA; (ii) the central region of ORF1 (723 nt, absent in the I-ΔAsu construct) does not contain the signals required in *cis* for correct localization; (iii) the Loc+ phenotype of an *I factor* RNA deficient in ORF1 can be rescued upon the supply of sufficient amounts of ORF1p provided in *trans*.

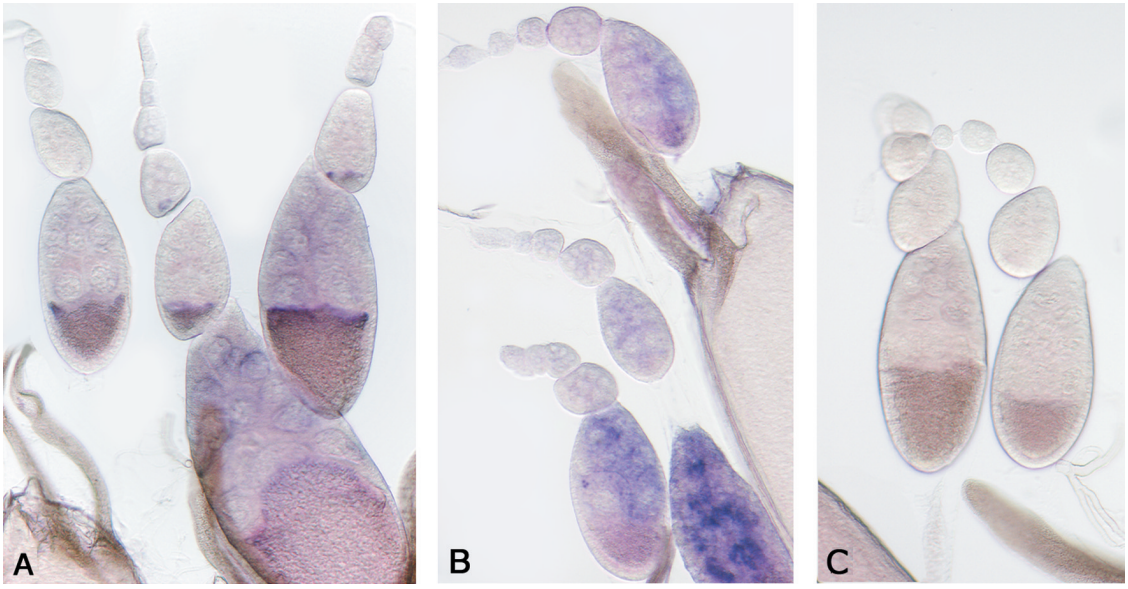

Figure 5. The *cis*-acting signal located within ORF2 drives the localization of a heterologous RNA. *In situ* hybridizations, using a *lacZ* probe, show that *lacZ* transcripts are not detected in the ovaries of control JA flies (**C**). In the ovaries of 313 flies, *lacZ* transcripts are scattered in the cytoplasm of the 16 cells of cysts (**B**). In 313/loc ovaries, the chimera *lacZ*/loc transcripts display a typical Loc+ phenotype like *I factor* transcripts (**A**).

DISCUSSION

Results obtained from actively transposing *I factors* showed that ORF1p (23) and full length *I factor* RNA co-localize according to a dynamic pattern (Loc+) exclusively in permissive oocytes (Figure 1a, panels A–D). The spatial and temporal co-localization of ORF1p and *I factor* RNA coincide with the expected place and time where the *I factor* is known to transpose (i.e. during the development of permissive young female germ cells), and suggests that during this period both molecules visualized here are a part of the transposition-related RNP complexes, expected early intermediates in the NLR retrotransposition process. Transcriptional activity is repressed in the oocyte and most maternal products synthesized during oogenesis are produced by the nurse cells and transported to the oocyte (33). Full-length *I factor* transcripts and ORF1p are both detected in the cytoplasm of very early oocytes, indicating that the RNA is transported to this cell immediately after synthesis in nurse cells, and that at least the first ORF of the bicistronic transcript is translated and transported at that time. The dynamic localization of these products coincides with nuclear migration to the anterior pole of the oocyte, indicating that both the nucleus and a fraction of *I factor* products remain in close contact during oogenesis. Some *Drosophila* morphogenes (proteins and/or RNAs), such as the *bicoid* or *gurken* mRNAs, follow similar re-localization patterns via motor proteins associated with the minus end of microtubules (dyneins) at mid-oogenesis (34–37). This similarity suggests that *I factor* products also localize using minus-end microtubule associated motor proteins. Through an ORF2p loss-of-function mutant (element I-HAO1-fsO2), we observed that ORF2p is not required for localization. Since localization of mRNAs is often coupled to translational control (38,39), one can hypothesize that ORF2p is synthesized as a late product, only after ORF1p and the RNA have reached the anterior pole of the oocyte where they meet cellular factors required for the differential translation of ORF2p from the bicistronic transcript. ORF1p and the RNA become undetectable after stage 10b of oogenesis when nuclear breakdown is known to occur. It is possible that the translation process of ORF2 results in ORF1p release and degradation as the newly synthesized ORF2p is activated to proceed with the subsequent steps of reverse transcription and integration in the genome.

Using deletion derivatives of a tagged *I factor* we have identified a specific region of 552 nt within ORF2 containing a signal required for proper localization of both ORF1p and *I factor* RNA (Figures 2–4). This fragment fused to sequences of the *lacZ* bacterial gene promotes a similar localization of the *lacZ* transcripts, suggesting that cellular cargos or cargo-adaptor proteins can directly recognize it. Transport signals are often carried by stable stem-loop structures (34,40). Several robust stem loops predicted in the 552 nt region of the *I factor* RNA are targets to test for interactions with ORF1p and for their involvement in localization.

The evidence that both ORF1p and the 552 nt signal in the RNA are implicated in the localization process of *I factor* products was obtained through the observation that an element deficient in ORF1 (I-ΔAsu) localizes very poorly in oocytes but recovers a full Loc+ pattern when a full-size ORF1p is provided in large amounts in *trans* (Figure 6). The extremely low efficiency of the *cis*-acting localization signal in the ORF1-mutant element (Figure 6A), compared with its high capacity to promote anterior pole localization when fused to *lacZ* RNA (Figure 5A), could be either due to the presence of the truncated ORF1p and/or due to a difference in the secondary structure resulting from a different sequence environment. The rescue of a full Loc+ phenotype when I-ΔAsu was complemented by ORF1p suggests that a possible role of ORF1p is to act as a molecular chaperone directing RNA folding into a localization-competent structure, probably via site-specific binding, either inside and/or outside of the 552 nt stretch.

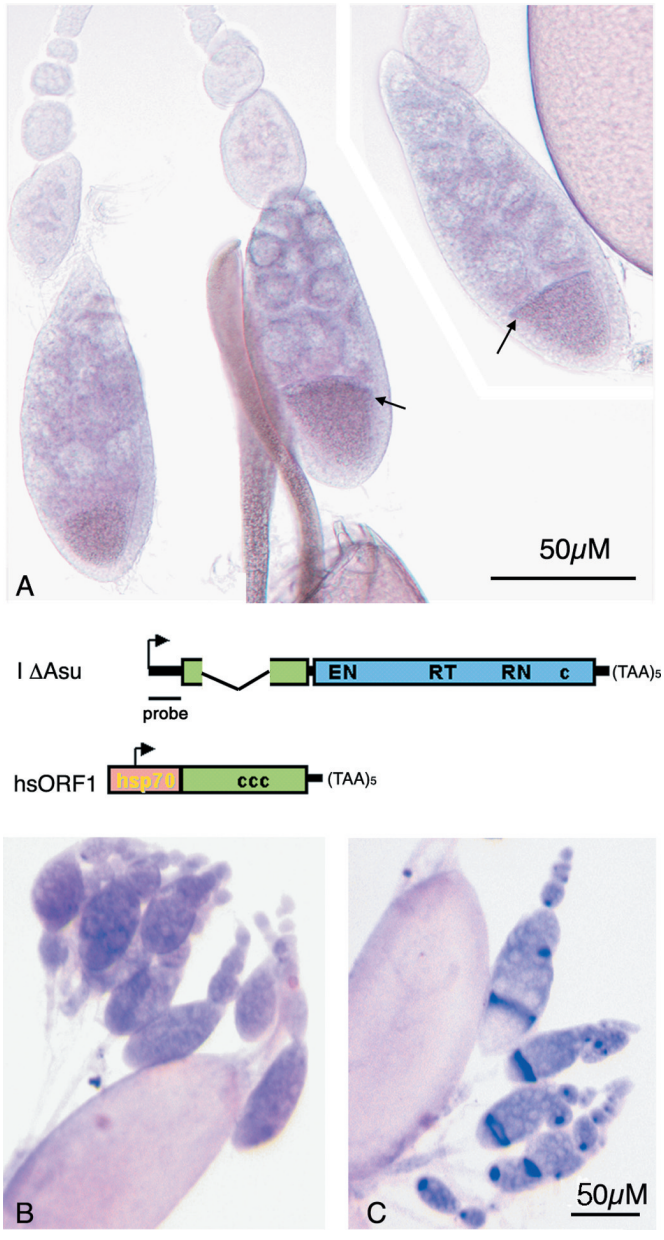

Figure 6. ORF1p can act in *trans*. The transcripts of the I-ΔAsu element, deleted in ORF1, do not accumulate in oocytes of flies issued from crosses of Is37 (line containing I-ΔAsu) with JA reactive flies, but some weak signals may be observed occasionally at the anterior pole of stage 10 oocytes (arrows in **A**). A heat-shock treatment does not affect the Loc− phenotype of I-ΔAsu (**B**). In the ovaries of females resulting from crosses between Is37 and HH16 (carrying the *hs*ORF1 construct), when overproduction of ORF1p is induced by a heat-shock treatment, a typical Loc+ localization pattern is observed (**C**).

Such a property is evocative of the chaperone Ncp7 protein which controls the conformational state of HIV RNA (41). The sequence-specific binding of the ORF1p of NLRs is a matter of debate. Assays performed with the human L1 p40 produced in heterologous systems have also led to the conclusion that binding does not require a specific sequence (18). However, p40 extracted from teratocarcinoma cells (in which L1 presumably transposes) specifically binds to two sites in the ORF2 RNA sequence (42), suggesting that some host dependent post-translational maturations may be essential for the full expression of ORF1p properties.

Our complementation data as well as previous experiments (20,24) indicate the importance of the localization process (Loc+) for efficient *I factor* transposition. Defective human L1 elements are poorly complemented in *trans* (43,44) and a mechanism called *cis*-preference has been proposed to account for low complementation efficiency. This mechanism refers to functional proteins that preferentially remain associated with the RNA molecule from which they are synthesized, favoring the specific mobilization of the progenitor element (45). Early experimental data suggested that the *cis*-preference mechanism might also apply to the *I factor* since previous attempts to mobilize defective *I factor*s led to very low complementation efficiencies, even under conditions of protein overexpression (20,24). Noticeably the elements used in those experiments did not contain the localization signal (ORF2 552 nt region). We have observed that the efficiency of complementation can be significantly improved by the use of deleted elements endowed with the localization signal and the appropriate supply of interacting proteins (ORF1p in our experiments). Another case of successful complementation when these conditions were met resulted in the rescue of the total transposition capacity of the non-functional element I-Δnuc (Loc+, deleted in the endonuclease domain) by a construct overexpressing a full-size ORF2p (46). Hence, at least part of the complementation efficiency to mobilize defective *I factor*s seems to depend on the localization signal interacting with one or more molecular partners. Under such conditions, genetic complementation analyses of *I factor* remain a powerful tool to continue dissecting the molecular components required for retrotransposition.

Analysis of the *Drosophila* genome has recently revealed the presence of 27 NLR families with two ORFs (47–49), but very little is known of the *in vivo* expression of any of these *Drosophila* NLRs. The expression in *Drosophila* cultured cells of ORF1p–GFP fusions from five of these *Drosophila* NLRs has shown a diversity of intracellular localizations: some of them enter the nucleus, but Doc or *I factor* ORF1p-GFP do not (50,51). The *in vivo* expression pattern of *Drosophila* NLRs other than *I factor* is still undocumented except for the transcripts of the Doc and HetA elements in *Drosophila* oocytes (52,53), but probably because the conditions of their retrotransposition are unknown, this pattern was not correlated to transposition. However, further investigations may reveal that the localization process described here for *I factor* RNPs reflects a more general situation.

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