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

Maria del Carmen Seleme, Olivier Disson, Stéphanie Robin, Christine Brun, Danielle Teninges* and Alain Bucheton

Institut de Génétique Humaine, C.N.R.S., 141 rue de la Cardonille, 34396 Montpellier, France

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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
transposition products in *Drosophila*. *I* factor transposes specifically in the oocytes of females (called SF) issued from mating males carrying active *I* factors (inducer or I strains) with permissive females lacking active *I* factors (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* factors [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* factors during mobilization co-localize with a specific cytoplasmic pattern (Loc+), exclusively in permissive oocytes. By analyzing the expression patterns of mutated/deleted *I* factors 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 hsORF1 and I-ΔAsu, respectively, and were established after P-element mediated transformation of the reactive strains wK 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 Smal–EcoRI fragment from plasmid pBTK2, at the end of ORF1 in construct pCaSpeR/I-HAO1 cut with Hpal–EcoRI (Figure 2). To obtain construct pCasPer/I-HAO1-fsO2, an 8 nt oligomer was ligated to EcoRV-digested pCasPer/I-HAO1. The I-ΔAsu construct contains an in-frame AsuII deletion within ORF1 and was derived from pB54, a plasmid containing a complete *I* factor cloned into vector pUCHneo. Construct hsORF1 was described previously (24). To obtain construct DF313/loc, a lacZII *factor* fusion, we generated the 552 nt localization fragment by PCR using primers 5'-GTATCTAGAAGCTACCTAGCAGC-sense and 5'-GACTAGTGGCTTTGTAGTGCGG-antisense, digested it with Xbal/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 pUCHΔAs2-3 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 hsORF1 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'-TTACCATACGACGTCCAGAG (sense) which overlaps the HA tag and 5'-GATCAGATCCTGATCTTTAGA (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 digoxigenin 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 factors 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 gerarium, 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 gerarium 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 gerarium (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 factors (E), nor in the ovaries of I flies, where active I factors are silenced (F). (b) Loc+ pattern of I-HAO1-fo2. 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 factors 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-HA01-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-HA01-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 RNA to the female germ line. The expression of the heterologous RNA was assessed by immunostaining using an antibody specific to the lacZ enzyme. The results showed that the expression of the heterologous RNA was dependent on the presence of the 552 nt segment, as was the case for the endogenous I factor RNA. These findings suggest that the 552 nt segment drives the localization of the heterologous RNA.
or construct I-loc, or with the unmodified DF313 transform reactive flies either with DF313/loc, creating the phenotype of Loc

alpha4-tubulin gene 67C that drives the expression in oocytes. The chimera lacZI 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 lacZI 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-Asu (24), which lacked 723 nt between the two AsuII restriction sites of ORF1. I-Asu 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-Asu 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-Asu 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-Asu and full-length ORF1p into the same oocyte. For this, we performed reciprocal crosses between line Is37, containing the I-Asu element and a line containing the construct hsORF1 (HH16 line) (24). In the construct hsORF1 the expression of ORF1p is driven by the heat-shock promoter hsp70. 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-Asu RNA (Figure 6B). In contrast, when Is37/HH16 females were subjected to heat treatment, a large proportion of I-Asu 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-Asu 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.
oogenesis. Some Drosophila I factor fraction of the pole of the oocyte, indicating that both the nucleus and a fraction of maternal products coincides with nuclear migration to the anterior pole of the oocyte. The dynamic localization of these maternal proteins is transported at that time. The localization of these maternal ORFs (ORF1 and ORF2) is expected to proceed with the subsequent steps of reverse transcription and integration in the genome. 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 I factor RNA are implicated in the localization process of ORF1p suggests that a possible role of ORF1p is to act as a molecular chaperone directing RNA folding into a stable stem-loop structure resulting from a different sequence environment. This 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. 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 I factor 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.
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 matu
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