High-Frequency Retrotransposition of a Marked *I* Factor in *Drosophila melanogaster* Correlates With a Dynamic Expression Pattern of the ORF1 Protein in the Cytoplasm of Oocytes

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ABSTRACT

To study the expression of the *I* factor, a non-long-terminal-repeat retrotransposon responsible for I-R hybrid dysgenesis in *Drosophila melanogaster*, we have tagged the ORF1 protein (ORF1p) by inserting the HA epitope in its N-terminal region. In transgenic flies, this modification is compatible with a high rate of autonomous transposition and allows direct estimation of the transposition frequency. *I* factor transposes in the germline of females (SF) that are daughters from crosses between I strain males (which contain active copies of the *I* factor) and R strain females (which do not). We analyzed the expression pattern of ORF1p by indirect immunofluorescence. Its expression correlates with retrotransposition. During oogenesis ORF1p appears unexpectedly as a cytoplasmic product, which accumulates with a specific pattern into the oocyte. A comparison of the expression patterns under conditions that modify the transposing activity of the element clarifies some aspects of *I*-factor functioning in the transposition process.

NON-long-terminal-repeat (non-LTR) retrotransposons are a large class of repeated DNA sequences widely distributed among eukaryotes. They are devoid of terminal repeats and contain an A-rich sequence at the 3′ end. Transposition occurs by reverse transcription of a full-length RNA as has been shown for the *I* factor, a non-LTR retrotransposon of *Drosophila melanogaster* (Chaboissier *et al.* 1990; Jensen and Heidmann 1991; Pelisson *et al.* 1991). The coding strand of most such elements contains two long open reading frames (ORFs). ORF2 shows strong similarities with retroviral reverse transcriptases (reviewed in Eickbush 1994). In contrast, the role of the ORF1 product (ORF1p), is unclear. It is only poorly conserved among non-LTR elements, and in some, such as R2Bm, it is absent altogether. It has often been considered as a retroviral *gag* gene equivalent, because a cysteine-rich motif observed in the ORF1p of invertebrate and plant non-LTR elements can be aligned with the CCHC motifs of the retrovirus nucleocapsid proteins. The ORF1p of the mammalian LINE-1 non-LTR retroelements (*L1*) lacks these cysteine-rich motifs. Moreover there are no other convincing similarities to the capsid or the matrix proteins encoded by retroviral *gag* genes. The ORF1p encoded by *L1* elements is required for transposition in cell culture (Moran *et al.* 1996). It is abundantly expressed in some human and mouse carcinoma cell lines where it occurs in large multimeric cytoplasmic complexes associated with *L1* RNA, but this expression has not been correlated with transposition (Martin 1991; Martin and Branciforte 1993; Hohjoh and Singer 1996, 1997). The ORF1p of the *D. melanogaster I* factor is also essential to transposition because transposition is abolished by an in frame deletion removing 241 amino acids from the *I* factor ORF1p (I. Busseau, unpublished data). When expressed in a heterologous system, ORF1p can also form multimeric complexes and shows a binding affinity for nucleic acids *in vitro* (Dawson *et al.* 1997). However, the role of such complexes in transposition is not clear, and to date nothing is known about the expression pattern of the ORF1p in conditions where transposition is actually occurring.

The main interest in the *I* factor, which is responsible for the I-R system of hybrid dysgenesis in *D. melanogaster*, is that high levels of transposition can be induced experimentally. Reactive (R) strains of flies carry only incomplete and inactive copies of this element, whereas inducer (I) strains contain in addition a limited number of active copies (Bucheton *et al.* 1984). *I* factors are stable in I strains but transpose actively in the germline of hybrid daughters (called SF females) from mating I males and R females. Typically SF females are nearly sterile: they lay a normal number of eggs, but only a fraction of these hatch. Later when the females age, the proportion of hatching eggs increases (Picard 1971; Bucheton 1978). Females produced by the reciprocal

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cross (I females mated to R males), called RSF, are fertile and exhibit lower frequencies of *I*-factor transposition (reviewed in Busseau *et al.* 1994).

A full-length 5.4-kb transcript, specifically synthesized in the ovaries of SF females, is the transposition intermediate of the *I* factor (Chaboissier *et al.* 1990). This RNA is transcribed from an internal polymerase II promoter contained in the first 186 nucleotides (nts), which also contain elements required for correct regulation of the *I* factor (McLean *et al.* 1993; Udomkit *et al.* 1996). The transcript might also function as a bicistronic messenger (Bouhidel *et al.* 1994). The two ORFs of the *I* factor are capable of encoding proteins of 426 and 1220 amino acids, respectively. ORF1p exhibits three zinc finger domains (type CCHC). ORF2p has similarities to endonucleases (Feng *et al.* 1996), reverse transcriptases, and RNase H (Fawcett *et al.* 1986; Abad *et al.* 1989). The endonuclease domain in N-terminal position of ORF2p has been shown to display endonuclease activity *in vitro* (D. Teninges and A. Bucheton, unpublished results) similar to that reported for the human *L1* elements (Feng *et al.* 1996).

Analysis of the molecular intermediates synthesized during transposition of *I* factors is difficult owing to the large number of products derived from the defective, variously truncated *I* elements present in all *D. melanogaster* strains, and so far, none of the proteins potentially encoded by *I* factors during transposition has been characterized *in vivo*. To address this problem we have tagged ORF1 with a sequence encoding the HA epitope. Using a polymerase chain reaction (PCR)-based method to estimate the frequency of transposition we show that the marked *I* factor transposes autonomously at high frequency. ORF1p, visualized *in vivo* using antibodies raised against the HA epitope or against a C-terminal peptide from ORF1, is cytoplasmic and accumulates in the oocytes of SF females. These tools allow, for the first time, an analysis of the spatial and temporal expression pattern of the ORF1p from a non-LTR element at the time of retrotransposition.

MATERIALS AND METHODS

Drosophila strains and fly care: Unless otherwise stated, the standard strong reactive (R) strain used in this work was line JA (*y* and *w*). For the *in situ* immunostaining experiment, line Charolles (wild type) was the second strong R strain and lines O/O (*v* and ry^{506}) and HJ30 (wild type) were the two weak R strains used. The standard strong Inducer (I) strain was w^{1118}. All crosses and lines were maintained at 23° with short generation times such that flies of each generation were never derived from mothers older than 1 wk. All strains used in the experiments are M in the P-M system of hybrid dysgenesis; thus, transposition events cannot result from *P*-element activity (reviewed in Engels 1989).

Plasmids and nucleic acid manipulations: The HA epitope was chosen for its similarity in charge and amino acid composition to the recipient region, the commercial availability of specific monoclonal anti-HA antibodies, and minimal cross-reactions of this antibody with fly proteins. The sequence used to construct *I*-HA-O1 derives from pI407, a plasmid containing a complete and functional *I* factor (Bucheton *et al.* 1984; Pritchard *et al.* 1988). The complete *I*-factor sequence was transferred into the plasmid pSP6T7-19 (Boehringer-Mannheim, Mannheim, Germany), creating the plasmid pSPT-19/I, which was used as a template to construct the HA-tagged element. All the *I*-factor sequences correspond to Fawcett *et al.* (1986; Flybase ID: FBgn0001249; Genbank accession no. M14954). Three synthetic oligonucleotides used as PCR primers directed our cloning strategy: (i) 5′AACAATCATGACAaattacccatacgacgtcccagattacgctaca GACCCACCAAAC3′ (sense); (ii) 5′gcatgtcgacggatccactagtaaatCAGTACCACTTC 3′ (sense); (iii) 5′TGTTAACGGTTAGGGTTTTGTGTAGAG 3′ (antisense).

Uppercase letters are *I*-factor sequence nts. In primer i, underlined nts correspond to the *Bsp*HI site and contain the first translation start codon of ORF1 located at position 186 of the *I* factor. Lowercase nts correspond to the HA-tag insertion sequence. Primer ii contains a multi-cloning site (MCS; lowercase) including the *Sal*I, *Bam*HI, and *Spe*I sites (underlined) and the first 12 nts of the 5′ end of the *I* factor. Primer iii anneals to the end of ORF1 (nts 1469–1495). The PCR products obtained from the pairs of primers i-iii and ii-iii were digested by *Bsp*HI and fragments containing the HA sequence and the MCS-5′ end of *I* were ligated. The ligation product was amplified using the primers ii and iii. A *Sal*I-*Bsp*MI restriction fragment (nts 4–380) was then isolated and substituted for the homologous fragment in pSPT19/I to create the plasmid pSPT19/I-HA-O1 (Figure 1). We verified that the new sequence derived from ligation of the PCR products (nts 1–500) was not otherwise mutated in the coding region. The *Sal*I-*Bsp*MI fragment from the PCR product ii-iii (without the HA tag) was similarly treated to obtain a control plasmid pSPT19/I. The *Pvu*II-*Eco*RI fragment from each plasmid was introduced into the *Stu*I-*Eco*RI sites of pCaSpeR4 (Pirrotta 1988) to create the control pCaSpeR4/I and the tagged pCaSpeR4/I-HA-O1 plasmids. Plasmids were transformed into *Escherichia coli* BL21(DE3) (Novagen) for expression of ORF1 under the control of the T7 promoter.

Construct pCaSpeRhs/ORF1 was made by inserting a 1.3-kb *Hpa*II-*Hpa*I Klenow-treated fragment containing ORF1 from pI407 into the *Hpa*I site of pCaSpeRhs (Thummel and Pirrotta 1992). The plasmid pCaSpeRhs/HA-O1 was constructed by exchanging the *Hpa*II-*Hpa*I fragment of pCaSpeRhs/ORF1 with the homologous fragment from pSPT19/I-HA-O1.

***P*-element-mediated transformation and transgenic lines:** Transformation was done according to Spradling and Rubin (1982), using the plasmid pUChsΔ2-3 (Flybase ID: FBmc-0000938) as the source of transposase (see Engels 1989). Plasmids pCaSpeR4/I and pCaSpeR4/I-HA-O1 were microinjected into embryos of the R strain JA. Different independent homozygous transgenic lines (HT1 to HT3 for Homozygous-Tagged, and HC1 to HC3 for Homozygous-Control lines) were established from orange-eyed G_1 flies and maintained by sibling crosses. To establish lines in which the transgene was preserved in males at each generation (Male-lines) G_1 heterozygous transgenic sons of G_0 males were selected and crossed to R strain females (T1 to T3 for Tagged and C1 to C4 for Control-untagged heterozygous lines). Male-lines were maintained by crossing at each generation heterozygous transgenic males (orange-eyed) to R strain females.

***In situ* hybridization to salivary gland chromosomes of larvae:** Polytene chromosome squashes and *in situ* hybridization were performed as described in Ashburner (1989). A fragment corresponding to nts 1–1500 of the ORF1 of the *I* factor was amplified by PCR and used as a probe. The plasmid p3w1.9 harboring a fragment of 1.9 kb of the *white* gene was used as

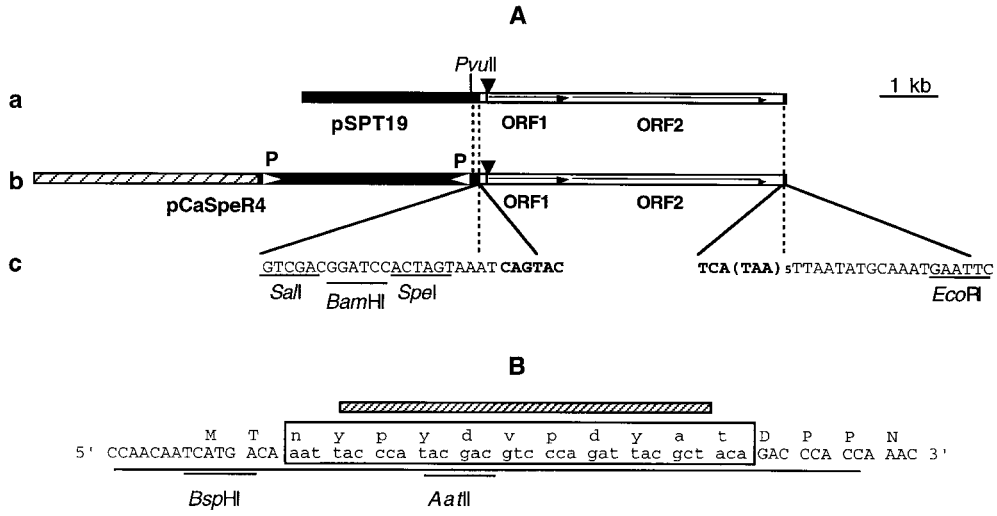


Figure 1.—Tagging the 5′ end of *I*-factor ORF1 with a sequence encoding an epitope from the influenza virus hemagglutinin (HA tag). (A) Structures of pSPT19/I and pSPT19/I-HA-O1 (a) and of pCaSpeR4/I and pCaSpeR4/I-HA-O1 (b). Open boxes represent the sequences from the complete *I* factor originating from pI407 (Bucheton *et al.* 1984). Positions of ORFs are indicated as thin arrows within the boxes. The black triangle indicates the position of HA tag within pSPT19/I-HA-O1 and pCaSpeR4/I-HA-O1. In (a), the black box represents the sequences from the vector pSPT19. In (b), the black box represents the bacterial sequences from pUC19, and the striped box represents the sequences from the mini*white*$^+$ gene (transcribed from left to right). White arrowheads indicate the positions of *P*-element sequences. Shown in (c) are the nucleotide sequences at the ends of the *I* factor (boldface characters) and the nucleotide sequences adjacent to them (standard characters), up to a *Sal*I site at the 5′ end, and up to an *Eco*RI site at the 3′ end. Restriction sites are underlined. (B) Sequence of the region of ORF1 containing the HA tag. Within the box and in lowercase letters is the inserted tag sequence. A striped box marks the exact limits of the epitope HA. The oligonucleotide sequence used as PCR primer (i) is underlined. An *Aat*II site was generated in the tag to facilitate further identification.

a technical control for hybridization. Probes were labeled with 11-dUTP-biotin by nick translation (Boehringer Mannheim). Three to four male larvae per transgenic line were analyzed to determine the mean number of euchromatic *I*-HA-O1 copies per line.

Transfection of Drosophila cultured cells: The pCaSpeRhs/HA-ORF1 vector was transfected into *D. melanogaster* Schneider line 2 (S2) cells. Cells were grown to 3/4 confluence in fresh Schneider medium supplemented with 10% fetal calf serum (Gibco, Paisley, U.K.) at 23°. For transient expression, 5 μg of plasmid DNA was transfected into cells by the calcium phosphate precipitation method (Ashburner 1989). The *hsp70* promoter was induced 3 days after transfection by two 30-min incubations at 37° with intervening 45 min at 23°. For SDS/PAGE, cells were harvested 3 or 4 hr later and extracted. For immunostaining, a suspension of cells was laid and dried on a slide after appropriate dilution. Fixation, blocking, and antibody incubations were done as described below for the immunostaining of ovaries.

PCR method to estimate the transposition frequency of marked *I* factors: An HA-specific PCR amplification product (1300 nts) is obtained from flies carrying the epitope when using the HA-tag sequence as the plus strand primer i and the end of ORF1 as the minus strand primer iii (Figure 3A). In Male-lines, the transgene *I*-HA-O1 should not transpose from its original insertion site. As the original transgene insertion site is linked to the [w^+] eye color marker, transposition could be checked by PCR analysis of Male-lines descendants devoid of the transgene (white-eyed). When males from Male-lines T1, T2, and T3 were crossed to R strain females, HA-specific amplification signal was obtained only from flies inheriting the transgene (orange-eyed), not from the white-eyed progeny, confirming that the *I*-HA-O1 element does not transpose in males. The orange-eyed daughters from such crosses are SF females because they contain one active copy of *I*-HA-O1 capable of transposing in their germline. To analyze transposition events in these SF daughters, they were crossed individually to (*white*) R strain males and samples of their white-eyed sons were tested by PCR. An HA-specific amplification signal in these sons reveals the presence of at least one transposed copy of *I*-HA-O1 giving a minimal estimation of the actual transpositon rate in the germline of each SF mother. The DNA from individual flies was prepared according to Gloor *et al.* (1993). PCR conditions were 30 amplification cycles at 95° for 1 min, 57° for 1 min, 73° for 2 min, 30 sec.

Immunoblotting: SDS/PAGE was performed according to standard procedures (Laemmli 1970; Schägger and von Jagow 1987). Proteins were transferred to nitrocellulose membranes and incubated with specific monoclonal anti-HA.12CA5 (Boehringer Mannheim). Primary antibody binding was detected with anti-mouse peroxidase conjugate and ECL chemiluminescent substrate (Amersham, Pharmacia Biotech Europe, Freiburg, Germany).

Immunofluorescence and confocal laser microscopy: Ovaries were dissected in 0.01% Triton X-100, phosphate buffered saline (PBS), fixed for 20 min in 4% formaldehyde, 0.3% Triton X-100, PBS, and rinsed with 0.5% bovine serum albumin, 0.3% Triton X-100, PBS (PBT). Blocking was done with 1.5% horse serum in PBT for 20 min. Ovaries were incubated with the primary antibody anti-HA.12CA5 (1:500 dilution) overnight at 4° and washed with PBT. Bound primary antibodies were detected using fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibodies (Vector Labs, Inc., Burlingame, CA) (1:100 dilution) for 2 hr at room temperature. After rinsing with four changes of PBT, ovaries were mounted using Vectashield medium (Vector). Rabbit polyclonal antibodies against the C-terminal nine amino acids of ORF1 protein (1:10.000 dilution) were detected with secondary Cy3- or FITC-conjugated anti-rabbit IgG antibodies (Vector; 1:150 or 1:300 dilutions, respectively). To facilitate the penetration of antibodies into late-stage egg chambers already synthesizing the vitelline membrane, the fixation step was modified for some samples by incubating ovaries for 25 min in 75% heptane, 1% formaldehyde, and 1× PEMs (0.1 m PIPES, 2 mm $MgSO_4$, 1 mm EGTA) and washing once in methanol:heptane and three times in methanol. Mouse monoclonal anti-α-tubulin antibodies, clone B5-1-2 (Sigma, St.

Louis; 1:2000 dilution) were used to control the accessibility of late-stage egg chambers to antibodies.

Samples were viewed in a Nikon microscope fitted with UV channels for fluorescent emission and in a confocal microscope. The confocal unit (Molecular Dynamics, Inc., Sunnyvale, CA) consisted of a laser scanning confocal microscope (Sarastro 2000) equipped with a 25-mW argon laser, an upright Nikon microscope equipped with Silicon Graphics Iris 35W/D, and Indigo workstations. Fluorochromes were excited at 488 nm (FITC) and 514 nm (Cy3). Images were processed using IMAGE SPACER and Adobe Photoshop programs.

RESULTS

Construction of an HA-tagged *I* factor and establishment of transgenic lines: We introduced the HA tag into the ORF1 N-terminal region of a complete *I* factor as described in materials and methods. The tagged element, called *I*-HA-O1, and an untagged control (*I*), both transferred into the transformation vector pCaSpeR4, were introduced by *P*-element-mediated germline transformation into R strain flies. Lines homozygous for the transgenes HT1 to HT3 (for Homozygous Tagged lines) and HC1 to HC3 (for Homozygous Control lines) were established and maintained by sibling crosses. Because *I*-factor transposition does not occur in males, we established and maintained heterozygous "Male-lines" where the transgenes, either *I*-HA-O1 (T1 to T3 lines) or the untagged *I* control (C1 to C4 lines), were conserved as single copies (see materials and methods).

Transgenic lines behave as typical inducer strains: The most easily detectable effect of *I*-factor activity is the sterility of SF females that can be scored as the hatching percentage of their eggs. To test the capacity of transgenic males to induce the typical I-R sterility syndrome of SF females, males from HC and HT lines at generation 8 (G_8) after transformation and males from HT lines at G_{22} were crossed with R females and the hatching percentages of the eggs laid by their F_1 daughters were scored. As a control for normal fertility, we determined the hatching percentages of the eggs from RSF F_1 daughters from the reciprocal cross (thus having the same genotype as the corresponding SF females). Transgenic males from either HC or HT lines produced moderately sterile daughters since the hatching percentages of their eggs were lower than 65% (Figure 2A). Such hatching percentages are similar to those of the eggs laid by SF daughters of IR crosses using weak I males (Bucheton *et al.* 1976). Although levels of fertility were higher than those of SF females derived from crosses of R females to standard I males, they were significantly lower than those of RSF females (Figure 2A). The hatching percentages of the eggs laid by SF females increased as they aged (data not shown), which is a typical feature of SF sterility (Picard 1971; Bucheton 1978). The fact that similar results were obtained with both the HC and the HT lines suggests that the presence of the HA epitope in *I*-HA-ORF1 was not affecting the capacity of this element to induce SF sterility. The *I* factors used in these experiments are derived from pI407, an element that has been shown to induce high sterility levels (Pritchard *et al.* 1988); however, it is not known why they behave differently.

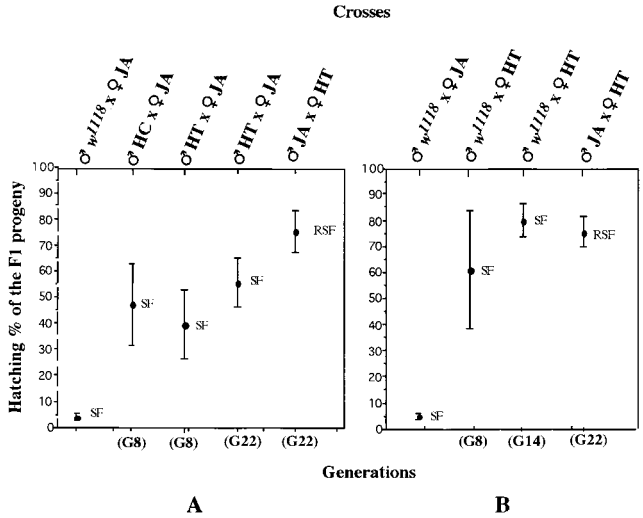


Figure 2.—Transgenic lines behave as inducer strains. (A) Inducer tests showing the capacity of transgenic males from homozygous HA-marked (HT) lines and from unmarked (HC) lines to induce hybrid dysgenesis. Individual males from transgenic lines were crossed with R females of the JA stock and the fertility of their young SF daughters (3–4 days old) was estimated as the hatching percentage of their eggs. Young RSF daughters from the reciprocal cross (in which each R strain male was crossed to a pool of three HT females) served as a nondysgenic fertility control of this genetic background. The fertility of young SF daughters from crosses of R females to independent standard I males of the w^{1118} stock was determined. G_8 and G_{22} indicate the generation number after transgenesis at which males were tested for inducer capacity. Independent scores of egg-hatching percentages per line were pooled to get a mean value for each type of transgenic line, HC and HT (dots) (11 HC males were tested at G_8: 5 HC1, 4 HC2, and 2 HC3; 6 HT males were tested at G_8: 2 HT1, 2 HT2, and 2 HT3; 20 HT males were tested at G_{22}: 7 HT1, 6 HT2, and 7 HT3; 15 pools of HT females were tested at G_{22}). The Mann-Whitney *U*-test was used to compare means: HC(8) *vs.* RSF(22), $z = -3.685$ ($P = 0.0002$); HT(8) *vs.* RSF(22), $z = -3.503$ ($P = 0.0005$); and HT(22) *vs.* RSF(22), $z = -4.783$ ($P = 0.0001$). Means obtained for HC(8), HT(8), and HT(22) were not significantly different. (B) Reactivity level of transgenic females from HT lines. Tests were done at generations G_8 and G_{14} by crossing pools of three HT females to each standard I male and determining the hatching percentage of the eggs laid by their young daughters (at G_8 two pools of females from the HT1 and HT2 lines and one pool of females from line HT3 were tested; at G_{14} three pools of females for HT1, three for HT2, and two for HT3 were tested). At each generation the independent scores of egg-hatching percentages were grouped, and means were calculated (dots). The reactivity of the R females was also controlled by crossing these females to standard I males (as in A). The hatching percentages of the eggs laid by the young RSF daughters of R males and HT females is also shown (as in A). Bars are standard deviations for each set of values.

Reactivity of females is a cellular state permissive for

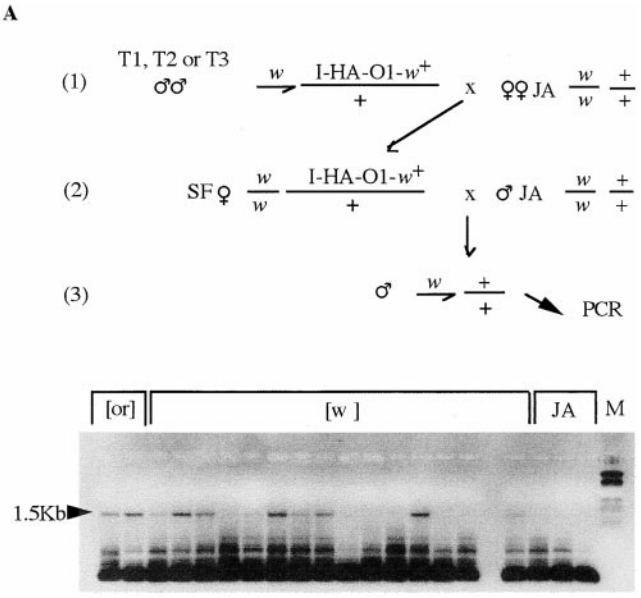
I-factor transposition (reviewed in Busseau *et al.* 1994). It is a state that is gradually lost over generations after *I* factors have been introduced into an R stock by crosses (Picard *et al.* 1978) or transgenesis (Pritchard *et al.* 1988). We tested the reactivity of transgenic lines by crossing G_{14} and G_{22} HT females to standard *I* males and scoring the hatching percentage of the eggs laid by their progeny. We observed that after a few generations, HT females (originally derived from the R strain) were no longer reactive. The hatching percentages of eggs laid by RSF daughters of crosses between R males and HT females were our control level of normal fertility (Figure 2B). Similar results were obtained with HC lines (data not shown).

To verify that the marked element had transposed in HT lines, we performed *in situ* hybridization to polytene chromosomes of HT individuals at G_{25} using an ORF1 *I*-factor probe (see materials and methods). For HT1, HT2, and HT3, we observed a mean number of 4, 7, and 12 euchromatic insertions of the *I*-HA-O1 element, respectively. As expected, the T1, T2, and T3 Male-lines had conserved a single transgenic copy of the tagged element localized, respectively, in chromosome arms *2*R, *3*L, and *2*L.

All these results indicate that HT and HC lines behave as typical inducer strains: they contain several copies of the *I* element, males exhibit inducer activity, and females are no longer reactive.

The tagged *I* element transposes autonomously at high rates in SF females: A primary advantage of the marked *I*-HA-O1 element is the ability to selectively follow its mobilization over the background of endogenous *I* elements in the genome. We have designed a PCR-based technique described in materials and methods to estimate the transposition frequency of the single *I*-factor copy contained in Male-lines. Figure 3B summarizes the results. The estimated frequencies for lines T1, T2, and T3 were, respectively, 0.76, 0.61, and 0.20 transposition events/gamete/generation. Pooling together these values, the mean transposition frequency of the marked *I* factor is estimated at about 0.5 transposition events/gamete/generation, which was approximately the rate observed for natural *I* factors (Picard 1976). For methodological reasons, these values are minimal estimations of the actual transpositon rate (see materials and methods). Position effects might account for the significant difference in transposition rate between T3 and the other two lines.

In SF females, ORF1p appears very early in oogenesis and accumulates specifically in the cytoplasm of oocytes: Drosophila ovaries are composed of parallel bundles of developmentally ordered egg chambers, called ovarioles. Oogenesis begins in the germarium at the anterior tip of each ovariole, where 2 or 3 stem cells divide asymmetrically to produce a cystoblast. Cystoblasts divide with incomplete cytokinesis to form cysts of 16 cells interconnected by ring canals. One cell becomes the oocyte, while the other 15 develop into polyploid nurse



B

Male line	T1	T2	T3
PCR positive/ total:	2/5 4/4 3/3 4/5 5/5 4/5 1/5 4/5 5/5	3/5 4/4 1/4 2/4 3/5 3/5 1/4 5/5	1/4 0/5 3/5 0/5 0/5 2/5 0/5 2/5
Sum	32/42	22/36	8/39
Frequency of transposition	0.76±0.13	0.61±0.16	0.20±0.12

Figure 3.—Frequency of transposition of the *I*-HA-O1 element (using PCR). (A) Top: Crosses performed. Males from each of the transgenic Male-lines T1, T2, or T3 were crossed with R females of the JA stock (1). Daughters with orange eyes are SF females in which transposition occurs in the germline. For each Male-line, eight or nine SF females were crossed individually with R males of the JA stock (2). The white-eyed progeny do not contain the original transgene but may contain transposed copies of the tagged element. A sample of three to five white-eyed sons (3) of each SF female was isolated and processed for PCR detection of transposed copies of the tagged element. Bottom: Analysis on a 1% agarose gel of PCR amplification products of DNA from single males. Lanes [or], product from orange-eyed males carrying the transgene; lanes [w], product from white-eyed males; lanes JA, product from JA negative controls; lane M, molecular weight marker (lambda phage DNA digested by *Hin*dIII). (B) For each SF mother, the ratio of PCR-positive sons to the total analyzed was recorded independently. Individual results per SF female were pooled to estimate the transposition frequencies and the standard deviations. Any positive PCR amplification recorded corresponds to one or more transposition event per individual genome analyzed.

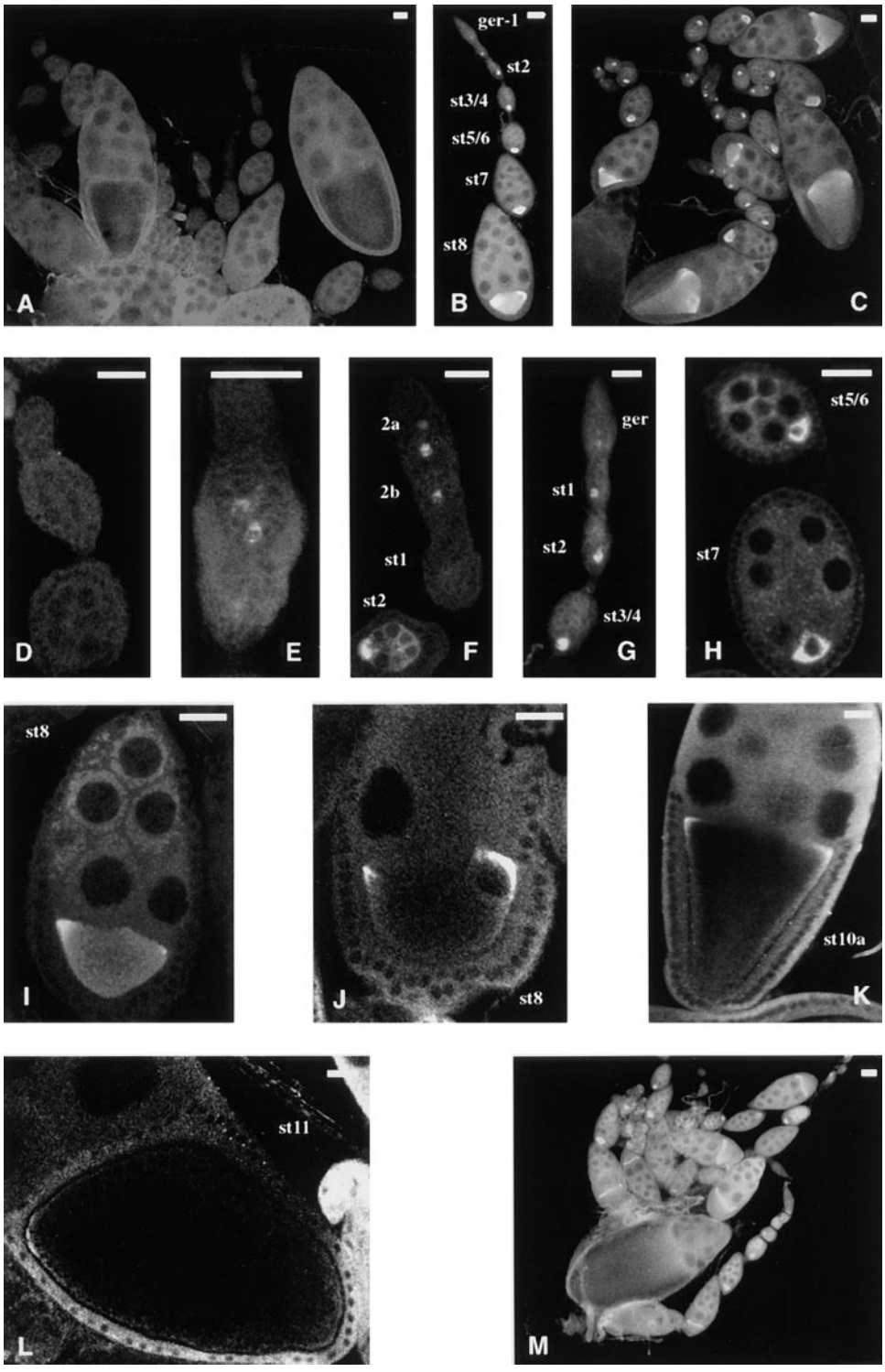


Figure 4.—Expression pattern of ORF1p during oogenesis in SF females. Indirect immunofluorescence detection on whole-mount ovary preparations was done using anti-HA antibodies (from A to L) and using anti-ORF1-C-terminal-nonapeptide antibodies (in M). Bound antibodies were revealed with (A to L) horse anti-mouse antibodies or (M) goat anti-rabbit antibodies, coupled to FITC. Fluorescent staining was visualized by confocal microscopy. Images are "look/through extended-focus" projections of six optical sections of 1 μm. The developing gradient of ovarioles (early-anterior to late-posterior) and the antero-posterior polarity of egg chambers are both positioned from top to bottom. (A and D) General views of an ovary and a germarium, respectively, of a JA reactive female showing the fluorescence background level. In B, C, and E–L SF daughters that were analyzed (HT-SF) were derived from HT males crossed either to Charolles (B and G) or JA (C, E, F and H–L) strong reactive females. In M, the SF ovary shown was prepared from a daughter of a standard I male (w^{1118}) crossed to an R strain female. No nuclear label of the ORF1p was detected in germ or somatic follicle cells (B, C, E, F, and H–M). (B) A fluorescent signal specific for the HA-ORF1p is present in the cytoplasm of HT-SF oocytes from the anterior tip until stage 10 of oogenesis. An enlarged view of the anterior region of this ovariole is shown in G. (C) HT-SF ovary in which all ovarioles are similarly labeled. No signal is apparent in the stage 11 egg chamber (bottom left). (E) Germarium of an HT-SF ovariole. HA-ORF1p is expressed early in oogenesis: beginning in region 2a of the germarium where it accumulates in the pro-oocyte cytoplasm. (F, G, and I) Germarium, stage 1, 2, 3/4, and stage 8 egg chambers of HT-SF ovarioles. In these images HA-ORF1p appears as a dispersed signal in the cytoplasm of nurse cells and as a strong and dense signal in the oocyte. (H) The cytoplasmic product of ORF1 appears concentrated posteriorly and around the nuclei of oocytes until stage 7 egg chambers. (I–K) From stage 8 egg chambers, the HA-ORF1p concentrates at the anterior cortex of the oocyte, surrounding the nucleus repositioned at this stage at the anterior-dorsal pole of the cell (J). (K) In stage 10a egg chambers the HA-ORF1p is still located at the anterior pole of the oocyte and a fluorescent dispersed granular label is detected in the surrounding follicle cells. (L) At stage 10b egg chambers, the label disappears progressively from the anterior cortex of the oocyte. Follicle cells are still labeled. (M) The expression pattern of the C-terminal region of the wild-type ORF1p is identical to that revealed by the N-terminal anti-HA antibodies in transgenic HT-SF females. Developmental stages are divided according to Spradling (1993). ger, germarium; st, stage. Bars, 20 μm.

cells. Each cyst is then surrounded by a sheath of somatic follicle cells forming together an egg chamber. The development of an egg chamber has been divided into 14 stages (reviewed in Lin and Spradling 1993; Spradling 1993; Theurkauf 1994; Grünert and St. Johnston 1996).

We have studied the expression pattern of the HA-ORF1p during oogenesis by indirect immunofluorescence on whole-mount ovary preparations using anti-HA monoclonal antibodies. The general background level of fluorescence was determined on ovaries of R strain females (Figure 4, A and D). Fluorescence was never detected in nuclei (Figure 4, B, C, and E–M). In the ovaries of young (about 3 days old) SF daughters of homozygous HT males mated with R females, an intense fluorescent label was apparent in all developmental stages from region 2 of the germarium, to stage 10 egg chambers. From the earliest stages, the signal was clearly concentrated in the cytoplasm of the oocyte (Figure 4, B and F), although a dispersed label of much lower intensity could be observed in the cytoplasm of nurse cells (Figure 4, C, H, and I). From stages 2 to 7, the immunofluorescent label occupied mostly the posterior pole of oocyte around the central nucleus. In stage 8 oocytes, when the microtubules rearrange anteriorly and the nucleus migrates to the antero-dorsal region (Theurkauf 1994), the HA-ORF1p also relocalized and concentrated at the anterior cortex of the oocyte (Figure 4, C and I–K). In the antero-dorsal pole, the signal surrounded the oocyte nucleus and was strongest in the vicinity of the microtubule network (Figure 4J). From stage 10b, at the time of rapid transfer of the cytoplasm from nurse cells to the oocyte and at the beginning of ooplasmic streaming, the fluorescent signal disappeared progressively (Figure 4, C and L). Using antitubulin antibodies, we determined that this disappearance was not resulting from a reduced accessibility of the antibodies to the oocyte due to the vitelline membrane, which starts to be deposited at stage 10 (not shown). ORF1p was detected in the cytoplasm of follicle cells surrounding the oocyte from stage 9 egg chambers onward (Figure 4, K and L). The same pattern was seen in SF females derived from the three HT lines as well as in SF daughters from R strain females crossed with Male-line males containing a single copy of the marked *I* element.

An antibody raised against a synthetic nonapeptide corresponding to the C terminus of ORF1 was also used to label ovaries of young SF daughters from R strain females crossed either to HT males (data not shown) or to standard I males (Figure 4M). The expression pattern observed in both types of SF females was identical to that observed with the anti-HA antibody recognizing the N terminus of ORF1p in transgenic SF females. Thus the particular dynamic pattern observed for ORF1p is not an artefactual effect of the HA-tag insertion in the *I*-HA-O1 element.

R strains are classified as weak or strong on the basis of the degree of sterility of their SF daughters (Bucheton *et al.* 1976). The SF daughters of HT males crossed to females of four different R strains, two strong and two weak (see materials and methods), were examined and in each case the spatial and temporal immunostaining ORF1p pattern was essentially the same (Figure 4, B and G). The expression pattern described above is thus a general one for all of the reactive stocks studied. However, the reactive type of the mother exerts an influence on the intensity of ORF1p expression because SF daughters of weak R strains had correspondingly weaker immunofluorescent signals (data not shown).

SF female sterility is partially cured by aging (Picard 1971; Bucheton 1978). To study the effect of aging on the expression of ORF1p, we performed immunostaining using both antibodies on ovaries of 14- to 16-day-old SF daughters from HT males mated to R strain females. At this age, these SF females had recovered a nearly normal fertility (65–70 hatching percentage compared to 45–55% at the age of 2–3 days). We observed three types of ovarioles: (i) ovarioles with the same level and type of ORF1p expression as in young SF females, (ii) mosaic ovarioles containing both ORF1p-negative and -positive egg chambers (Figure 5, A–C), and (iii) ovarioles completely devoid of fluorescent signal (data not shown). The expression pattern observed

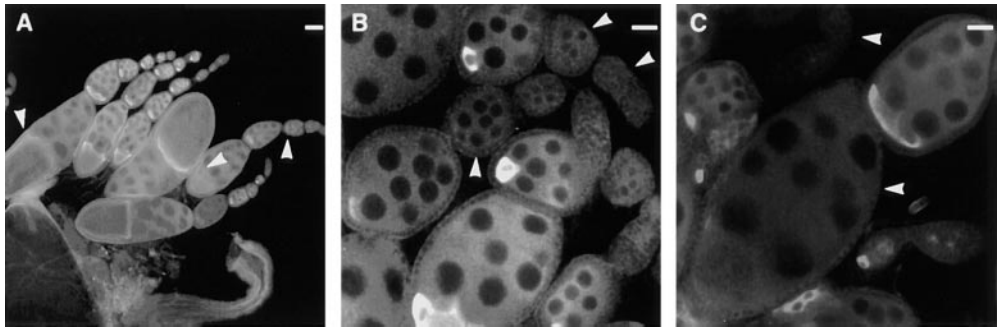


Figure 5.—*I*-HA-O1 expression in old HT-SF females. Immunostaining conditions are as in Figure 4. Images are look/through extended focus projections of six optical sections of 1.5 μm. (A) Ovary of a 16-day-old HT-SF female. The intensity of expression and the localization of HA-ORF1p is the same as in young SF females. ORF1p is absent from some egg chambers (arrows) at any stage of development: (B) Germarium and stages 2 or 3; (C) Stage 9. Bars, 20 μm.

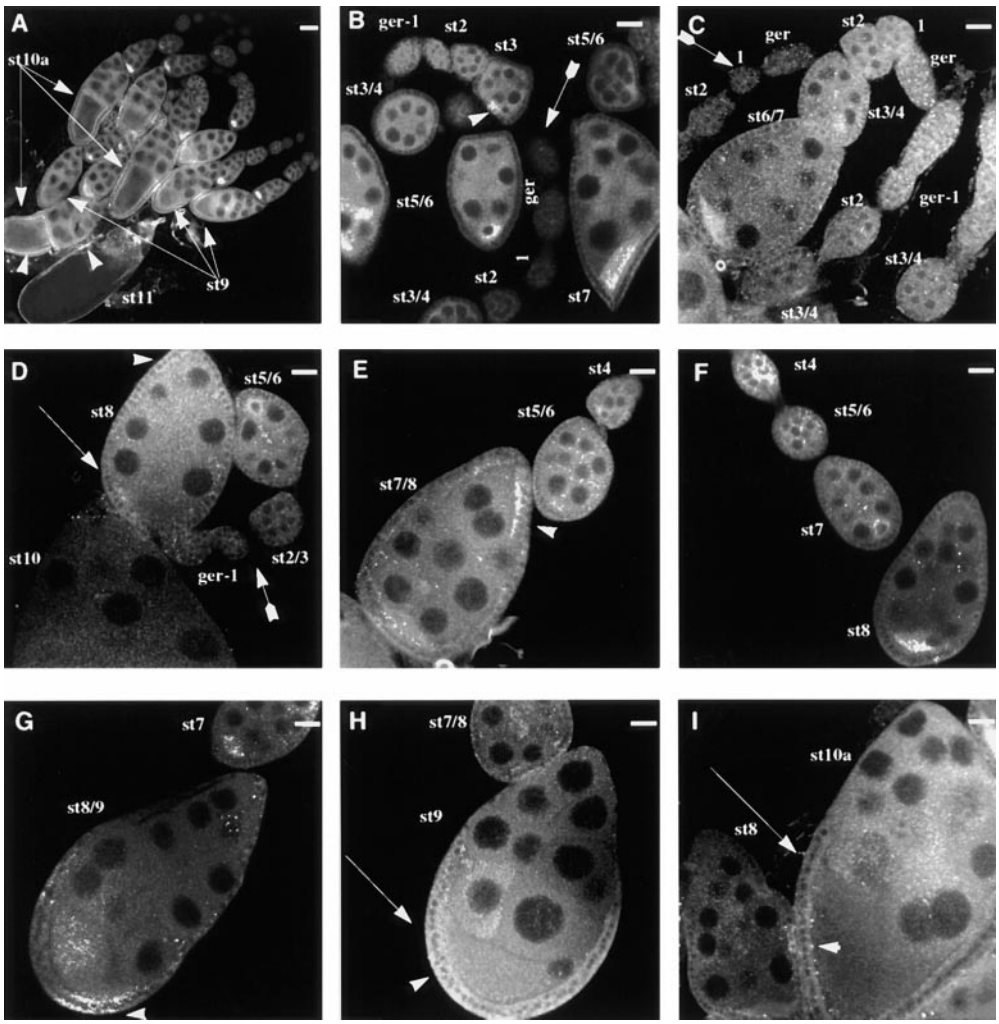


Figure 6.—Expression of ORF1p in the ovaries of RSF daughters from crosses of R males of the JA stock to HT females. Immunostaining conditions are as in Figure 4. Images are look/through extended focus projections of nine optical sections of 1.5 μm. ORF1p is cytoplasmic in RSF ovaries. (A) General view of an ovary. Note the different expression pattern of ORF1p with respect to SF female ovaries in Figure 4C. Arrows point to egg chambers from stages 9 onward, where ORF1p is not detected in the cytoplasm of oocytes (as in D, H, and I). (B–I) Diverse ovarioles showing the very heterogeneous expression pattern of ORF1p. (B and C) In some germaria and early stage egg chambers (1/2/3) the fluorescent label appears uniformly distributed among all cells without a specific accumulation in the oocytes. Tailed arrows point to other early-stage egg chambers where no expression of ORF1p is apparent (in D, also). The ORF1p label is concentrated in the cytoplasm of oocytes from stages 3/4 to stages 7/8/9 of oogenesis (B, D, and F, strong; or C, E, G, and I, weak and diffuse signals). At stage 8, ORF1p does not migrate to the anterior cortex of the oocyte (F)and in general, the fluorescent signal is weak and appears dispersed in the cytoplasm of this cell (E, G, and I). From stage 8/9 egg chambers, ORF1p is heterogeneously expressed in the cytoplasm of nurse cells and the signal appears with a granular aspect rarely seen in SF females (D, H, and I). Arrowheads point to groups of follicle cells that appear intensely labeled at any stage of oogenesis from germarium to stage 10b/11 egg chambers, either surrounding the oocyte (A, G, H, and I) or the nurse cells (B, D, and E). Developmental stages are divided according to Spradling (1993). ger, germarium; st, stage. Bars, 20 μm.

in aged SF daughters from strong R mothers was clearly distinct from that observed in young SF daughters from weak R mothers, although both types of SF flies are nearly fertile.

Ovaries of females from either the standard I strain or the transgenic HT lines were also analyzed by immunostaining and no labeling of ORF1p was detected (data not shown). This is consistent with the fact that *I* factors are repressed in I strains and that *I*-HA-O1 behaves like a typical *I* factor.

The expression pattern of ORF1 is heterogeneous in RSF females: In RSF females, which are fertile, *I* factors transpose at lower rates than in SF females (Picard 1976). We analyzed by immunostaining the expression of ORF1p in ovaries of young RSF daughters from R strain males crossed to females from either transgenic or standard I strains. In such RSF daughters, ORF1p expression differed from that seen in SF females:

1. At the beginning of oogenesis (region 2 of the germarium to stage 2/3 egg chambers), either there was no detectable expression in germ cells (tailed arrows in Figure 6, B–D), or the ORF1p signal appeared evenly distributed among all cells with no specific concentration in the oocyte (Figure 6, B and C). Some germaria presented very bright granular signals that may correspond to expression in somatic cells (follicle stem cells and prefollicle and interfollicular cells) localized near the outer surface of the germarium (Figure 6C).
2. In some mid-stage egg chambers (stages 4–7), the signal accumulated in the oocyte cytoplasm with variable intensity (Figure 6, B–G).
3. In stage 8 oocytes, ORF1p was not concentrated at the anterior cortex and the label was in general diffuse and weak (Figure 6, E, G, and I); only rarely was it as intense as is shown in Figure 6F.

4. From stage 8/9 ORF1p generally disappeared from the cytoplasm of the oocyte (arrows in Figure 6, A, D, H, and I). In nurse cells, the ORF1p expression was heterogeneous and granular (Figure 6, D, H, and I).
5. In follicle cells an intense and granular ORF1p label occurred throughout oogenesis in apparently randomly distributed cell patches (arrowheads in Figure 6, A, B, D, E, and G–I).
6. Interestingly, a few egg chambers at stage 8/9 showed the ORF1p signal accumulated at the anterior cortex of the oocyte exactly as in SF females (data not shown).

Analysis of HA-ORF1 protein: The ORF1 of the *I* factor contains two putative translation start sites according to Cavener and Ray (1991) and Brown *et al.* (1994). The first one (AATCATG at position 183) is located 399 nts upstream of the second (AATAATG). The HA epitope was placed immediately downstream of the first AUG codon and its expression was immunodetected *in situ*, showing that this codon is actually a translation start site (at least in SF and RSF ovaries). The expected size for the full-length ORF1p is 48.5 kD and, with the HA epitope, 49.8 kD. When HA-ORF1 is under the control of the *hsp70* promoter and transfected into Drosophila cultured cells, a specific protein having the expected relative mobility for a full-length ORF1p (49.8 kD) was revealed in Western blots from cell extracts (data not shown), indicating that ORF1p is not processed by proteolytic cleavage in these cells. The immunolocalization of HA-ORF1p in these cells was exclusively cytoplasmic (data not shown) as in the germline of SF females.

DISCUSSION

Many attempts have been made to construct marked *I* factors that would be able to transpose at high frequency, but transposition rates of these elements were actually very low, usually less than 10^{-3} transposition event/gamete (Jensen and Heidmann 1991; Pelisson *et al.* 1991; Chaboissier *et al.* 1995; Busseau *et al.* 1997). In contrast, the HA-tagged *I* factor transposes at a frequency of about 0.5 transposition event/gamete, which is in the same range of magnitude as that reported for wild-type *I* factors (Picard 1976). The *I*-HA-O1 factor is a powerful tool allowing a direct estimation of the transposition rate by a PCR-based method described here, which can easily be applied to study the effects of genetic and epigenetic factors on transposition. This method is notably less time consuming than the genetic procedures previously used to estimate *I* factor transposition rates (Picard 1976).

The *I*-factor ORF1p is a cytoplasmic product: Models for non-LTR retrotransposition propose that ORF1p is a major component of ribonucleoprotein (RNP) complexes that would package the RNA transposition intermediate and ORF2 products. These complexes would allow *I*-factor products to enter the nucleus (see Busseau *et al.* 1994). We observed here by *in situ* immunostaining that the fluorescent signals tracing either end of ORF1p of the *I* factor colocalize in the cytoplasm of oocytes in which tranposition actually occurs at very high frequency. This suggests either that ORF1p is not cleaved or that its eventual cleavage products remain associated. This product is synthesized in the nurse cells and accumulates in the cytoplasm of the oocyte until its progressive disappearance along with the formation of the vitelline membrane (stage 10) and before the breakdown of the oocyte nuclear envelope (stage 14; Dävring and Sunner 1977). Cytoplasmic localization of ORF1p during transposition suggests that it might be mostly implicated in the transportation of the *I*-factor RNPs from nurse cells, where they are assembled, to the vicinity of the oocyte nucleus into which the integration complex would finally be addressed, with subsequent degradation of ORF1p. However, our data do not exclude the possibility that a small undetected amount of ORF1p, or a cleavage product of this protein, enters the nucleus as part of the integration complex. In this case, since the only predicted nuclear localization signal in the *I* factor lies within ORF2 (KKRKK at amino acid position 330) and since ORF2p is synthesized in much smaller amounts than ORF1p (Bouhidel *et al.* 1994), a possible nuclear uptake of this complex could be limited by the stoichiometry of ORF1p *vs.* ORF2p.

A transgenic construct, K160, in which the bacterial *lacZ* gene was fused in frame to part of the *I*-factor ORF1, was previously expressed in reactive, SF, RSF, and inducer context (Lachaume *et al.* 1992; Tatout *et al.* 1994). In contrast to our results, the *lacZ* activity of K160 was concentrated in the nuclei of germ cells from reactive and SF females only and no expression was found in follicle cells. This expression was not that of a functional *I* element but that of a construct encoding only amino acids 1–34 and 275–305 of ORF1p. It was thus lacking the CCHC motif shown by Dawson *et al.* (1997) to be required in the formation of macromolecular complexes, but not in nucleic acid binding. Our data demonstrate that the localization of this chimeric protein does not reliably reflect the actual subcellular localization of the complete functional *I*-factor ORF1p. Although it is currently accepted that the ORF1p of non-LTR elements assembles in RNP complexes that migrate to the nucleus where transposition occurs, we observe here that the ORF1p of an active *I* element remains cytoplasmic.

The *lacZ* temporal expression of K160 was similar to that of ORF1p reported here, indicating that the *I*-factor sequences contained in this construct (nts 1–290 and 1014–1104) are sufficient to define the timing of expression of ORF1 in the female germline.

The ORF1p localization in oocytes correlates with

active transposition: The *I* factor is known to transpose in the germline of SF females (Picard 1976; Bucheton 1990). By *in situ* immunostaining analysis we have shown that ORF1p exhibits a characteristic localization during oogenesis with a pattern that is sensitive to factors known to affect the frequency of *I*-element transposition (SF *vs.* RSF females; age of flies). In the vast majority of young SF female oocytes, ORF1p displays a precise dynamic pattern and accumulates at the anterior cortex of the cell. In these flies we have shown that the marked element transposes at high rates. The distribution and amounts of this product are different in RSF and in old SF females. Both types of females show higher fertility levels, lower transposition rates, and lower abundance of *I*-factor full-length transcripts compared to young SF females (Picard 1971, 1976; Bucheton 1978; Chaboissier *et al.* 1990). ORF1p appears to be less abundant in old SF females than in young SF females, with a complete absence of the protein in many egg chambers, but, when the protein is present, its localization is exactly similar to that observed in young SF females. This pattern of expression might be the result of a random transcriptional switch-off taking place at the beginning of oogenesis and determining the presence or absence of ORF1p in the developing oocyte. Then, in old SF females the relative number of oocytes where ORF1p accumulates at the anterior cortex is lower than that seen in young SF females. In RSF females the expression pattern of ORF1p is very different. This product is present in lower amounts, and in most egg chambers it exhibits a different pattern compared to SF females. However in a small fraction of RSF female egg chambers, ORF1p accumulates at the anterior cortex of oocytes as in young SF females. These observations argue in favor of a correlation of this particular pattern with effective transposition events and strongly suggest that the accumulation of ORF1p at the anterior cortex of the oocyte is required for *I*-factor transposition.

If the properties of ORF1p are not modified by differential processing in the SF and the RSF contexts, the different patterns of ORF1p accumulation seen in SF oocytes, as compared to the majority of RSF oocytes, indicate that the dynamic distribution and anterior accumulation of ORF1p in SF female oocytes are not intrinsic properties of this protein. This specific pattern may require the formation of complexes with other products of the *I* factor itself. A minimal concentration threshold of these elements (rarely reached in RSF females) might be necessary for a correct assembly of such complexes. Candidates constituting these complexes are the 5.4-kb full-length *I*-factor RNA and/or ORF2 products. This hypothesis can be further tested by studying the ORF1p expression pattern of elements diversely mutated in ORF2.

Involvement of the microtubule network in the functional localization of ORF1p: The slow phase of cytoplasmic transport selectively conveying nurse cell products to the oocyte depends on the microtubule network and starts as soon as the pro-oocyte is determined in region 2 of the germarium (reviewed in Mahajan-Miklos and Cooley 1994). This is likely to account for the transport of ORF1p from the nurse cells, where it is synthesized, to the oocyte where it accumulates from region 2 of the germarium. At stage 8, the posteriorly localized microtubule organizing center reorganizes to the anterior cortex of the oocyte. At this time, the nucleus migrates from a central position to the anterodorsal surface of the oocyte (Theurkauf 1994). Some maternal products, such as the *bicoid* mRNA (Berleth *et al.* 1988) or (transiently) the Staufen protein are also carried anteriorly at this stage, in a microtubule-dependent manner (Ferrandon *et al.* 1994). At stage 8, the ORF1p also relocalizes from the posterior to the anterior cortex of the oocyte in a movement that mimics that of *bicoid* mRNA. The dynamic localization of ORF1p during oogenesis, coincident with microtubule rearrangements and microtubule-dependent movements in the cells, strongly suggests that the molecular complexes including this protein are associated with the microtubule network of the egg chamber.

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