The full-length transcript of the I factor, a LINE element of *Drosophila melanogaster*, is a potential bicistronic RNA messenger

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Received February 3, 1994; Revised and Accepted May 6, 1994

ABSTRACT

The I factor of *Drosophila melanogaster* is a retrotransposon of the LINE superfamily. The I factor displays two non-overlapping open reading frames (ORFs) that have the potential to encode for a nucleic acid-binding protein (ORF1) and a reverse transcriptase (ORF2). Retrotransposition of the I factor has been demonstrated and a putative full-length RNA intermediate has been identified. No other transcript from functional I factor has ever been described, suggesting that the full-length RNA is also used as a messenger. Here we report that a bicistronic RNA which conserves the ORF1 – ORF2 organization of the I factor transcript is a template for ORF2 translation *in vivo*. We further demonstrate that the first AUG of ORF2 initiates translation, but efficiency of this initiation increases approximately 200 fold when ORF1 is deleted. Our results show that the I factor transcript may be used to translate both ORFs from their own initiation codons at different rates. Various mechanisms of translation are proposed.

INTRODUCTION

LINEs (Long Interspersed Nucleotide Elements), also known as nonviral or non-LTR retrotransposons, are a superfamily of transposable elements (1). In mammals up to 5% of the genome consist of the same LINE family: L1 (2). Although not as abundant in other organisms, LINE families are also found in protists, plants and insects (3). LINEs are devoid of terminal repeats but have an adenine-rich region at the 3' end (3). Full-length elements usually contain two separate or slightly overlapping long ORFs, one of which encodes a polypeptide similar to viral reverse transcriptases. Such an activity has been demonstrated for the *jockey* element from *Drosophila melanogaster* (4) and the *CRE1* element from *Crithidia fasciculata* (5). Direct evidence for retrotransposition is available for three different LINEs: the L1 elements of rat and mouse (6,

7), and the I factor of *D. melanogaster* (8, 9). In human, *de novo* insertions of LINE-1 elements have been found associated with hemophilia (10), or with tumors (11).

The I factor of *D. melanogaster* differs from all other LINEs by its ability to transpose at high frequency under conditions, named I-R hybrid dysgenesis, that can be experimentally induced. All *D. melanogaster* strains fall into one of two categories: inducer strains (I) which contain active I factors (~15 copies/haploid genome) and reactive strains (R) which do not (12). Crosses between I males and R females produce dysgenic females displaying germ-line specific abnormalities associated with I factor transposition such as low fertility and increased frequencies of mutation and chromosomal rearrangement.

Functional I factors are ~5.4kb long and contain two long ORFs (ref. 13 and 14; Fig. 1a). ORF1 is 1287bp long and potentially encodes a polypeptide featuring zinc-finger motifs. ORF2 is 3675bp long and potentially encodes a polypeptide displaying homologies with viral reverse transcriptases and ribonuclease HOs. Retrotransposition of the I factor has been demonstrated (8, 9) and a putative RNA intermediate has been identified (15). The fact that this full-length RNA is the only I factor transcript detected so far suggests that it is used as a messenger for synthesis of the I factor-encoded polypeptides. These polypeptides have not been identified, although expression of an ORF1-*LacZ* fusion gene has been observed in germ-line cells (16) where transcription and transposition occur. However, the location of ORF2, 53bp downstream from the end of ORF1 within the structurally bicistronic I factor transcript, raises the problem of its translatability. While genomic RNAs from caulimo- and hepadna-viruses are now considered as functional polycistronic messengers (17, 18, 19), little is known about the ability of cellular messengers to direct the translation of a downstream ORF. Recently ORF2 from mammalian LINEs have been shown to be translated from bicistronic transcripts *in vitro* and in cultured cells (20, 21).

Here we report that a bicistronic RNA homologous to the I factor transcript enables ORF2 translation *in vivo*, strengthening

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Figure 1. Fusion genes and their transcripts in Kc 167 cells. (a) Structure of the I factor is shown as deduced from the sequence reported by Fawcett *et al.* (13), modified by Abad *et al.* (14). Numbers refer to position within the sequence. The open arrow and the open boxes represent respectively the internal promoter and the ORFs of the I factor. Relative positions of domains of interest are indicated as NAB: nucleic acid-binding domain, RT: reverse transcriptase domain, RH: ribonuclease H domain. The first ATG of both ORFs and their position are indicated. (b) Structures of the bicistronic I-LacZ fusion gene and its monocistronic derivative. The black arrow and the black box correspond respectively to the promoter and the trailer of the *hsp70* gene. The stippled box represents the *LacZ* coding sequence. Deletion in pZ17TΔ1 is indicated as a dashed line. (c) Transcripts of the bicistronic and monocistronic fusion genes in transfected Kc167 cells. 5 μg of poly(A)⁺ RNAs isolated from cells transfected with either pZ17T alone or pZ17T and pZ17TΔ1 in equimolecular amount were hybridized with an antisense *LacZ* RNA probe (see Fig. 1b and Methods).

the idea that the full-length I factor RNA is not only the intermediate for transposition but also the messenger required for synthesis of the polypeptides catalysing this process.

MATERIALS AND METHODS

DNA constructs

For simplicity only a brief summary of how the constructs were made will be presented here. The full details of all of the cloning steps are available upon request. For construction of pZ17T (Fig. 1b), the first 160 bp of the I factor from pI407 (22), including the I factor promoter (23), were replaced by an *EcoRI*–*Bam*HI fragment from pHT 4 (24) containing the *hsp70* promoter. All I factor sequences after the *EcoRV* site at position 1570 were replaced by a *Xma*I–*Sal*I fragment from pMC1871 (25) containing the *LacZ* coding sequence. Finally, a *Cla*I–*Xba*I fragment from pHSREM1 (26) carrying the *hsp70* trailer was inserted at the 3' end of the *LacZ* sequence. pZ17TM (Fig. 3) is a pZ17T derivative generated by mutagenesis of the first I factor ORF2 ATG into AAG using the following oligonucleotide (mismatch to the wild type sequence underlined): CTCAC-AAAAGTCCCTAAC and the Oligonucleotide-directed *in vitro* Mutagenesis System 2.0 (Amersham) as recommended by the manufacturer. pZ17TΔ1 (Fig. 1b) and pZ17TΔ1M (Fig. 3) derive respectively from pZ17T and pZ17TM by deletion of all I factor sequences upstream from the *Hind*III site at position 1516. The β-glucuronidase reporter construct pGUS was obtained by replacing the *lacZ*-containing *Xba*I fragment from pCV2-βgal (J.L.Couderc) with the *Hind*III–*Eco*RI fragment from pRAJ260 (27) carrying the *E. coli* β-glucuronidase coding sequence (*uidA*). This placed *uidA* under the control of the *copia* promoter and the SV40 termination signal.

Cell culture and transfection protocol

The Kc167 *Drosophila* cell line was grown at 23°C in D22 medium supplemented with 2% fetal calf serum (FCS) (28).

Transfections were carried out by lipofection using the cationic lipid DOTMA (Boehringer Mannheim). 10⁶–10⁷ cells were transfected in 5 ml of CaCl₂-supplemented HBS (HBS: 150 mM NaCl, 20 mM Hepes pH7.4; 0.7 mM CaCl₂) containing 2.5 μg of pGUS plus the appropriate amount of one plasmid of the pZ series for a 3/1 molecular ratio, and 50 μl of lipid. After 6 hr at 23°C, 7 ml of D22 2% FCS were added. After incubation for another 12 hr at 23°C the medium was replaced with 10 ml of fresh medium and the cells further incubated for 48 hr. The cells were heat-shocked at 37°C for 1 hr, allowed to recover for 2 hr and heat-shocked again at 37°C for 1 hr. The cells were then harvested either for RNA isolation or β-galactosidase and β-glucuronidase assays.

RNA isolation and Northern blot analysis

RNA was isolated from ~10⁹ cells using the Quickprep Purification Kit (Pharmacia) under the conditions recommended by the manufacturer. RNA was subjected to electrophoresis through a denaturing agarose–formaldehyde gel, transferred to a nylon membrane and hybridized with a RNA probe as described in (29). A radiolabeled *lacZ* probe was synthesized by *in vitro* transcription using a recombinant pGEM-7Zf(–) plasmid carrying the 1.1 kb *Sac*I–*Eco*RI *lacZ* fragment from pMC1871.

β-Galactosidase and β-glucuronidase assays

The cells were scraped from the dish, washed twice with HBS, then resuspended in 100 μl of 0.25 mM sucrose, 10 mM EDTA, 10 mM Tris–HCl pH7.4 and lysed by 3 freeze/thaw cycles. The cell debris were removed by centrifugation (10,000 g, 15 min). An appropriate volume of supernatant was incubated in 1 mM 4-methylumbelliferyl β-D-galactoside (or β-D-glucuronide), 0.1% triton X-100, 10 mM 2-mercaptoethanol, 50 mM sodium phosphate pH7.0 with a final reaction volume of 0.2 ml. The reaction was incubated at 37°C for 30 min and terminated with the addition of 0.8 ml 0.2 M Na₂CO₃. Fluorescence was then measured with excitation at 365 nm, emission at 455 nm. β-

Figure 2. Transient translation of ORF2 *in vivo*. (a) Pre-blastoderm embryos were microinjected with either pZ17T or pZ17T Δ 1, then heat-shocked at various stages of development and treated to reveal β -galactosidase-expressing tissues. An individual was counted positive when β -galactosidase activity was detected in tissues expressing undetectable level of endogenous β -galactosidase activity. (b) Detection of β -galactosidase activity in the intestine of third instar larvae developed from embryos microinjected with either pZ17T or pZ17T Δ 1. A single cell expressing pZ17T is visible on the left picture.

galactosidase and β -glucuronidase activities are expressed in enzyme units. 1 unit is defined as the amount of enzyme that produces 1 nm of product/min at 37°C.

Microinjection and *in situ* detection of β -galactosidase activity

The reactive strain *Charolles* was used as recipient for the transient expression analyses. After a 1 hr pre-collection period, eggs were collected at 15–30 min intervals at 25°C. All other operations were carried out at 18°C. The DNA solutions (500 μ g/ml, 5 mM KCl, 0.1 mM sodium phosphate pH6.8) were injected into embryos as described by Zalokar (30). After injection, the embryos were transferred at 25°C and incubated for the desired time. After heat-shock (see above) non-devitelized embryos, as well as dissected larval and adult organs were stained as described in (31).

RESULTS

Synthesis of a RNA with the bicistronic organization of the I factor transcript

Expression of I factor has proven difficult to record *in vivo*: it is restricted to germ-line cells and controlled by maternal effect (16), full-length transcripts are observed at very low level only (15) and translation products are not detected by antibodies raised against synthetic peptides or a fusion protein (F.Evraert, personal communication). In order to assess the translatability of I factor ORF2 and to circumvent these shortcomings, a fusion gene was designed using *lacZ* from *E.coli* as a sensitive reporter gene and the transcription signals of the inducible *hsp70* gene from *Drosophila*. The resulting pZ17T construct (Fig. 1b) allowed us to analyze translation of ORF2 by β -galactosidase titration. From

Figure 3. Role of AUG1536 on ORF2 translation. Involvement of AUG1536 in translation initiation was assessed by point mutagenesis. Basal constructs (pZ17T and pZ17T Δ 1) as well as their mutagenized variants (pZ17TM and pZ17T Δ 1M: ATG1536 converted into AAG) are shown schematically. Each construct was cotransfected with a transcriptional fusion where the reporter glucuronidase gene from *E.coli* was placed under the control of the *copia* promoter. β -galactosidase and β -glucuronidase activities were titrated in cellular extracts from heat-shocked cells (+hs) or untreated cells (–hs). ORF2 expression is given in unit of β -galactosidase activity/milliunit of β -glucuronidase activity. Values represent averages from several independent transfections with the standard error computed for each condition. ND: not determined. For heat-shocked cells, relative expression to pZ17T is indicated in parentheses. In our experiments, heat-shock had no significant effect on the activity of the *copia* promoter (data not shown). The low level of β -galactosidase activity in untreated pZ17T Δ 1-transfected cells probably reflects a weak constitutive activity of the *hsp70* promoter.

the I factor pZ17T still retains the full ORF1, the inter ORF sequence, and the beginning of ORF2 with its first ATG at position 1536. Its expression is no longer restricted to germ-line cells. As a monocistronic control we constructed pZ17T Δ 1 by deleting all sequences from the I factor before the *HindIII* site at position 1516 (Fig. 1b). In this case the *hsp70* promoter-leader lies just upstream from the ORF2–*lacZ* fusion and drives the expression of a transcript with AUG 1536 from ORF2 as single potential start codon for *LacZ* translation.

Plasmids pZ17T and pZ17T Δ 1 were introduced by transfection into Kc167 cells. Forty-eight hours later, cells were heat-shocked and poly(A)⁺ RNAs were extracted and analyzed as described in Methods. The monocistronic construct pZ17T Δ 1 yielded the expected monocistronic RNA, while pZ17T gave a full-length RNA similar to the full-length transcript from I factor (Fig. 1c). Cotransfection with equimolecular amount of DNA showed that both transcripts had similar steady-state levels. No degradation product equivalent to the monocistronic RNA of pZ17T Δ 1 was found for pZ17T even when the film was overexposed (Fig. 1c): only the full-length bicistronic RNA was ever detected.

ORF2 is translated *in vivo* from the bicistronic mRNA

In situ stainings of transfected cells by X-Gal demonstrated that pZ17T and pZ17T Δ 1 can direct the synthesis of β -galactosidase (data not shown). To be sure of the ability of these constructs to express ORF2 *in vivo*, we microinjected them in embryos at pre-blastoderm stage. Embryos were then heat-shocked at various stages (5 hour old embryo, third instar larva, adult) and assayed for β -galactosidase. When a sufficient amount of DNA is injected all the cells receive and maintain potentially functional DNA for a least 10 hours (32). However, as development progresses, a lower percentage of the cells can still express the injected DNA. Transient expression of pZ17T and pZ17T Δ 1 were observed at all developmental stages investigated (Fig. 2a). Without an internal standard, the percentage of β -galactosidase-expressing

embryos may vary due to intrinsic variability of the experimental conditions. However we obtained clear-cut results: 6% of pZ17T-injected embryos were positive compared to 90% for pZ17TΔ1, suggesting that the level of β -galactosidase activity was lower when directed by the bicistronic construct. pZ17T- and pZ17TΔ1-directed β -galactosidase activities were observed in a variety of larval and adult organs such as intestine (Fig. 2b), malpighian tubules and ovaries. But once again, pZ17T-directed staining appeared always more sparse and/or faint than the pZ17TΔ1-directed one. From these results we concluded that ORF2 can be translated from the monocistronic messenger (probably by recognition of AUG 1536 as a start codon for translation) and from the bicistronic messenger.

Translation of ORF2 is initiated at internal AUG1536 albeit inefficiently

We showed that pZ17T and pZ17TΔ1 transcripts had similar steady-state levels in Kc167 cells after a heat-shock (Fig. 1c). However transient expression assays showed that ORF2 might be translated at a lower rate from the bicistronic RNA messenger. In order to clarify this point we quantified the β -galactosidase activity accumulated in Kc167 cells after transfection. As an internal control, a transcriptional fusion of the β -glucuronidase coding sequence from *E.coli* to the promoter of the *copia* transposable element, was cotransfected to monitor the efficiency of transfection in each plate of cells. β -galactosidase and β -glucuronidase activities were measured and the ratio β -galactosidase activity/ β -glucuronidase activity was calculated. Results are given in figure 3. Expression of ORF2 from the bicistronic construct pZ17T was strictly dependent upon a heat-shock and therefore upon the presence of the full-length bicistronic mRNA transcribed from the *hsp70* promoter. However, the rate of accumulation of β -galactosidase was approximately 200 fold higher with the monocistronic construct.

In order to know whether ORF2 translation was initiated at internal AUG1536 within the bicistronic transcript, we mutated AUG1536 into AAG, a codon unable to initiate translation (33). The monocistronic transcript pZ17TΔ1M, which bears this mutation, was unable to direct the expression of a β -galactosidase activity in transfected cells (Fig. 3), proving that AUG 1536 is a genuine start codon for translation. The same mutation within the bicistronic construct (pZ17TM) had the same effect (Fig. 3), indicating that ORF2 is translated from its own start codon within the bicistronic transcript, and that the presence of ORF1 prevents its efficient use.

The next point was to assess the ratio of ORF1 to ORF2 expression in the bicistronic construct. We fused lacZ with ORF1 and showed again that ORF1 expression was much more higher than ORF2 expression. But : a) this fusion gave not the same level of expression as pZ17Δ1 (we could assume that efficiency of the initiation codon was not the same for ORF1 and ORF2) and b) as we constructed two different translational fusions between ORF1 and lacZ we saw that one construct gave repeatedly three times more β -gal activity than the other. This suggested that each fusion protein had its own specific activity and that such specific activities might vary broadly. Therefore the levels of expression we recorded could be compared directly between our various ORF2 fusions, but could be compared neither between the two different ORF1 translational fusions, nor between ORF1 and ORF2 fusions as they gave different fusion proteins, the relative specific activities of which were unknown.

DISCUSSION

By placing the I factor under the control of an inducible promoter and fusing ORF2 with a reporter gene we were able to assess ORF2 translatability in different *Drosophila* tissues and in cultured cells. Our data show that ORF2 translation occurs from a bicistronic mRNA sharing the same ORF organization with I factor. The fact that there is no requirement for an internal transcription unit or an RNA splice to express the downstream ORF of the I factor is consistent with previous transcription studies: no RNA shorter than the full-length one has ever been detected (15). This result supports the idea that the full-length I factor transcript is not only the RNA intermediate for transposition but also the messenger required for synthesis of the proteins involved in this process.

As shown by mutagenesis experiments, ORF2 translation strictly depends on AUG 1536, the first AUG codon in ORF2 sequence. This indicates that there is no continuous translation from ORF1 to ORF2 through ribosomal frameshifting (3 frameshifts required to avoid intercistronic stop codons), but that ORF2 translation is initiated internally at the first ORF2 AUG. Recent results indicate that the translation of human LINE-1 element (L1Hs) ORF2 is most likely to be initiated internally (21). In addition, the ORF1-encoded polypeptide of L1Hs was identified *in vitro* and in cultured cells by using antibodies, but no ORF1-ORF2 fusion protein was ever detected (34). Moreover, *in vitro* ORF2 translation of a rat LINE-1 bicistronic transcript was demonstrated to be internally initiated (20).

Most translation initiations in eukaryotes occur according to the scanning mechanism proposed by Kozak (35). This model states that the 40S ribosomal subunit and its associated factors binds initially at the 5' end of mRNA and then migrates, stopping at the first AUG codon for initiating translation. However an increasing number of cellular and viral mRNAs are now known to initiate translation at internal AUGs (36). Four different models have been proposed to account for all the recorded exceptions and could explain internal initiation within the I factor transcript. These include (i) leaky scanning: the 40S subunit can bypass the first AUG if it does not lie in a favorable context for initiation (i.e. a purine at position -3 and a G at position +4) and reach another one further downstream (37), (ii) termination-reinitiation: after a stop codon the ribosome dissociates but the 40S subunit can remain associated with the mRNA, resume scanning and reinitiate translation at a nearby internal AUG (38), (iii) internal ribosome entry: the 40S subunit binds directly to an internal site and scans the RNA to reach the initiation site (39), and (iv) ribosome shunt: the 40S subunit first recognizes the 5' end of the RNA then 'jumps' directly to an internal binding site in order to initiate translation at the authentic AUG (40).

To reach the internal ORF2 initiator codon, the ribosomal subunit binding the 5' end of the I factor transcript must bypass 13 AUGs, many of them being in a more favorable context to initiate translation. This argues against the leaky scanning hypothesis. Additional experiments are however needed to discriminate between the three other mechanisms.

We examined the translation efficiency of ORF2 within both bicistronic and monocistronic mRNAs. Our data show that the efficiency of ORF2 translation decreases 200 fold when ORF1 is present without any correlated decrease in the steady-state level of transcripts. Such an inhibitory effect has been previously described for artificial bicistronic mRNAs (41). Thus, in our

experimental conditions internal initiation is significantly inefficient compared to initiation at the 5' end of the messenger. This suggests that I factor ORF2 polypeptide is synthesized at a lower rate than ORF1 polypeptide, and probably produced in minute quantities in the ovaries of the fly. However we cannot rule out the possibility that in this organ a trans-acting factor specifically enhances translation initiation at internal AUGs. Similar trans-acting factors have been described in yeast (42) and in plants (43).

A high ratio of ORF1 to ORF2 translation is consistent with our knowledge of the transposition cycle of LINEs. Models for LINEs transposition propose that reverse transcription occurs in a ribonucleoprotein particle containing full-length RNA and transposon-encoded polypeptides. Ribonucleoprotein particles with L1 RNA were identified in mouse and human cell lines (44, 45). In human cells, particles were also found associated with reverse transcriptase activity. In both cases, the ORF1-encoded polypeptide was identified as a major component of the particle but the ORF2 polypeptide escaped detection, suggesting that there are very few, possibly only one, ORF2 polypeptides per reverse transcription complex. Inefficient translation of a downstream ORF within a bicistronic mRNA may thus be responsible for the elusiveness of the LINEs reverse transcriptase.

ACKNOWLEDGEMENTS

We thank V.Irish, T.Nelson, N.Schultes, C.Day, and B.McGonigle for helpful suggestions on the manuscript. K.B. received fellowships from the Ministère de la Recherche et la Technologie, the Association pour la Recherche sur le Cancer and the Ligue Nationale Française contre le Cancer. This work was supported by grants from the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer and the Fondation pour la Recherche Médicale.

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