Suffix-specific RNAi Leads to Silencing of F Element in Drosophila melanogaster

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Separate conserved copies of suffix, a short interspersed Drosophila retroelement (SINE), and also divergent copies in the 3’ untranslated regions of the three genes, have already been described. Suffix has also been identified on the 3’ end of the Drosophila non-LTR F element, where it forms the last conserved domain of the reverse transcriptase (RT). In our current study, we show that the separate copies of suffix are far more actively transcribed than their counterparts on the F element. Transcripts from both strands of suffix are present in RNA preparations during all stages of Drosophila development, providing the potential for the formation of double-stranded RNA and the initiation of RNA interference (RNAi). Using in situ RNA hybridization analysis, we have detected the expression of both sense and antisense suffix transcripts in germinal cells. These sense and antisense transcripts are colocalized in the primary spermatocytes and in the cytoplasm of the nurse cells, suggesting that they form double-stranded RNA. We performed further analyses of suffix-specific small RNAs using northern blotting and SI nuclease protection assays. Among the total RNA preparations isolated from embryos, larvae, pupae and flies, suffix-specific small interfering RNAs (siRNAs) were detected only in pupae. In wild type ovaries, both the siRNAs and longer suffix-specific Piwi-interacting RNAs (piRNAs) were observed, whereas in ovaries of the Dicer-2 mutant, only piRNAs were detected. Further analysis of northern blots and SI nuclease protection assays showed that suffix-specific RNAi leads to the silencing of the relative LINE (long interspersed element), F element, and suggests that SINE-specific RNA interference could potentially downregulate a set of genes possessing SINE stretches in their 5’ or 3’ non-coding regions. These data also suggest that double stranded RNAs possessing suffix are processed by both RNAi and an additional silencing mechanism.

INTRODUCTION

Retroelements are ancient components of the genome, and are potential participants in some RNA-related regulatory mechanisms in the cell. The recent discovery of RNAi has extended our knowledge of such processes by uncovering mechanisms in which short RNA molecules are used by protein complexes for the recognition of specific nucleotide sequences that are important for the regulation of gene expression and also the formation of chromosomal structures [1]. In a landmark paper by Fire and colleagues [2], it was demonstrated that double-stranded RNA (dsRNA) is the trigger for RNAi silencing mechanisms. A number of mechanisms were subsequently described in which control of mRNA translation, the formation of heterochromatin structures, and the silencing of either mobile elements or unpaired DNA is mediated by RNAs as universal intermediates in homology sensing [3–5]. In some of these mechanisms, it has been postulated that mechanisms could serve not only as targets for silencing, but also as tools that provide RNA sequences for regulation.

Retroposition is an ancient genetic mechanism underlying the flow of information from RNA to DNA, resulting in the appearance of new copies of a corresponding sequence in the genome. Several classes of retroelements have now been detected during the last few decades: non-LTR mobile elements (or LINEs), LTR-elements that are closely related to retroviruses, and short retroelements (or SINEs). SINEs are too small to harbor a coding function, and for their transposition they use reverse transcriptases encoded by LINEs. Until now, the major portion of the SINEs described in different genomes are derived from either small structured RNA molecules of tRNAs or from 7SL RNA, which forms part of the ribosomal complex [6] and has an internal RNA polymerase III promoter [7]. Studies indicate that the internal promoter is not sufficient for in vivo transcription of a SINE, and that some control signals are required from the insertion site [8]. Hence, the majority of the SINE copies are transcriptionally inactive, i.e. non-functional fossil relics with respect to retropositioning [9]. Without selective pressure, they accumulate mutations or decay over the course of evolution. It is possible that a small part, or even a particular SINE copy (master or source gene), could be transcribed and its RNA potentially used for retropositioning [6,10]. In addition, although the mechanisms underlying retroposition remain unclear, several factors have been suggested to be important including the ability of the specific transcript to compete for association with the enzymatic machinery “borrowed” from LINEs for mobilization; and the length and homogeneity of the poly(A) stretch, which allows for effective priming [11]. The discovery of RNAi mechanisms, which are considered to be not only an ancient protective mechanism against retroelements, but

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are also regarded as a physiological tool for the regulation of gene activity [12–14], has made the study of transcription patterns of different retroelements more significant.

Suffix is an unexpected example of a short retroelement. Although it has a poly(A) stretch and a size that is typical of a SINE, it lacks the usual RNA polymerase III promoter and possesses a short open reading frame. Previously, suffix was found as a separate repetitive element with different sequences around (separate copies), as well as on the extreme 3' ends of some genes and also on the 3' ends of F and Doc elements [15–17]. Comparison of sequences of suffix and F elements led to the first demonstration that SINEs and LINEs share a common 3' sequence, possessing a small region of coding sequence, a poly(A) signal and a poly(A) site [16,18]. More recently, new examples of several pairs of SINES and LINEs from vertebrates and plants have been described [9,19].

It has recently been reported that in the Drosophila germline there are repeat-associated small interfering RNAs (rasiRNAs) that protect against retroelements by a novel RNA silencing mechanism [20]. These RNAs are distinguishable from siRNAs by their longer length (24–29 nucleotides, nt) and by the lack of the 2',3'-hydroxyl termini that are characteristic of miRNAs and siRNAs. Hence, silencing mechanisms involving rasiRNAs are distinct from the earlier described RNAi and miRNA pathways. In addition, they do not require Dicer-1 or Dicer-2 RNases and function through the PIWI protein family (Aub, Piwi, and Ago3). RNAs of 29–30 nt from testes that interact with Piwi proteins have also been described in mammals [21] and are known as piRNAs.

In our present study, we show that sense and antisense suffix transcripts are present during all stages of Drosophila development and are co-localized in the germline. However, suffix-specific siRNAs, the putative RNAi products, are detectable only in pupae. It is of interest that in the wild type ovaries, two classes of small suffix-specific small RNAs are present, siRNAs and piRNAs, as this suggests that an additional silencing mechanism targeting the suffix-containing transcripts is involved in the germ line. F element transcripts lacking the suffix stretch can also be detected in pupae. These data indicate that the suffix element is involved in developmentally regulated RNA-interference, which leads to silencing of the F element. Our current data also suggest a hypothetical novel mechanism, whereby the concerted silencing of genes occurs by RNAi targeting of a SINE sequence in the non-coding regions of mRNA sequences.

Figure 1. Relationship between the Drosophila F element and suffix. Neighboring DNA fragments (shown in brackets) were used for the preparation of both suffix-and F element-specific RNA probes for Northern analysis. doi:10.1371/journal.pone.0000476.g001

**RESULTS**

Transcriptional patterns of suffix and F elements

Previously, we demonstrated that suffix elements found in genes are present in a reversed polarity, and that the poly(T)-containing strand (minus strand) forms the last, very short intron and exon [16]. To further study the transcripts corresponding to both strands of this element in more detail, we performed Northern blotting analysis under stringent hybridization conditions. In these experiments, the signals should come from transcribed suffix sequences within genes, from F elements and also from transcripts of separate individual suffix copies. In our preliminary experiments, we found that under such conditions, signals from divergent suffix copies and Doc-like versions of this element could not be detected. Figure 1 depicts the neighboring regions of the F element that were used for the preparation of the strand-specific [32P]-RNA probes. For the preparation of these probes, we used suffix sequences and the upstream sequence of F elements subcloned into pGEM-vectors. The suffix-specific antisense [32P]-RNA probe would be expected to detect sense, poly(A)-containing transcripts generated from separate copies of suffix and the F element. We observed a major component of suffix-containing RNA bands (approximately 3500 nt) in both poly(A)+ and poly(A)− RNA samples during all developmental stages (Figure 2). Careful analysis revealed that this band was located above the smaller 18S rRNA and fragments of 28S RNA. The current databases contain one full-length, 3.6 kb cDNA (AC:AY71740), harboring the suffix element in the 3' non-coding sequence. This cDNA has a coding region specifying a reverse transcriptase that is homologous to the pilger element, a non-LTR Drosophila retrotransposon (AC:AJ270684).

It has been reported that Alu sequences are also detectable in both poly(A)+ and poly(A)− RNA preparations [11]. Interestingly, only in the Drosophila line under study (Oregon-Shostak), and not in several other lines that we tested throughout our analyses, did we observe a short poly(A)+ transcript of about 300 nt in length,
which is the size expected for a suffix full-length transcript. Moreover, this transcript was detected only in pupae. There are weaker transcripts of different lengths containing the suffix sense strand in poly(A)\(^+\) RNA preparations in embryos, larvae, pupae and flies. Our data on the nature of suffix-containing transcripts obtained with RLM-RACE (Ambion), will be described in a separate report.

A \([^{32}P]\)-sense-RNA probe reveals the presence of suffix antisense transcripts that would be predicted to come mainly from copies of these elements within genes. As expected, these transcripts are more prominent in poly(A)\(^+\) samples and their pattern and intensity changes during development. Some bands may also correspond to antisense transcripts generated from F element copies.

The same blots were re-hybridized with F element-specific \([^{32}P]\)-RNA probes after stripping of the previous probe. It has been believed for some time that the F element is transcribed for a short duration, and then only in embryos [22]. Northern analysis of F element is also hampered by the presence of very long transcripts in embryos, pupae and imago that cause smearing from the top of the gel [23]. However, modern techniques allow us to observe the transcription patterns of F element in more detail. In our current experiments, we observed a more discrete picture under stringent conditions of hybridization and washing. A strong 4700 nt band, corresponding to the full-length F element transcript [22], and a number of smaller poly(A)\(^+\) bands were revealed by the use of an F element-specific antisense probe in embryos, larvae, pupae and flies (Figure 3, right panel).

**Figure 3. Northern blot analysis of F element-specific transcripts.** Hybridizations were performed with F element-specific antisense or sense \([^{32}P]\)-labeled RNA probes. The labeling is as described for Figure 2. doi:10.1371/journal.pone.0000476.g003

SUFFIX sequences should be present at the 3’ ends of all poly(A)\(^+\) F element transcripts, but the corresponding bands are not visible against a background of much more abundant RNA molecules generated by the independent suffix copies (compare the left panel in Figure 2 with the right panel in Figure 3). Although the neighboring fragments of the F element were used for the preparation of the suffix- and F element-specific probes, it is clear that the patterns of hybridization for the antisense \([^{32}P]\)-probes corresponding to both the suffix and F elements are very different. This fact demonstrates that suffix is transcriptionally very independent from the F elements and that the signals generated by the separate suffix copies are much higher. Poly(A)-containing sense transcripts corresponding to F elements were also observed on a suffix-probed blot but only after a longer exposure (data not shown). Another difference is that, in the case of F elements, both sense and antisense transcripts are mainly polyadenylated. This again demonstrates that the essential portion of the suffix-specific transcripts is not generated from the F element.

There are known to be very long polyadenylated transcripts possessing F element and suffix sequences in embryos and flies, which probably come from regions of heterochromatin where the majority of these copies are found [15,22]. Both elements have another feature in common—their transcripts are generated from both strands. In addition, whereas, symmetrical suffix transcripts are present during all stages of development, symmetrical polyadenylated transcripts from F elements are present mainly in embryos and flies. In the current *Drosophila melanogaster* databases, there are only 8 suffix-containing ESTs that correspond to F elements. Hence, our present data on transcription patterns of suffix clearly indicate that the databases are still poor in suffix-containing transcripts.

**Figure 4. Expression patterns of suffix and F elements in *Drosophila* testis.** In situ hybridizations with DIG-labeled, strand-specific RNA probes were performed (Materials Methods). (A, B) The patterns revealed by suffix sense and antisense probes, respectively. (C, D) The patterns revealed by F element sense and antisense probes, respectively. Arrows indicate the transcripts detected in primary spermatocytes. doi:10.1371/journal.pone.0000476.g004

Sense and antisense transcripts of both suffix and F element are located in the same germline cells

To investigate the possibility that dsRNA may be formed by sense and antisense RNAs coming from both suffix and F element, we tested whether these transcripts are expressed in the same cells. We selected testes and ovaries for *in situ* hybridization analysis using the same RNA probes that were used in our Northern blots, but this time labeled with DIG (see Materials and Methods). We found that the suffix probes hybridized in the nuclei of primary spermatocytes (Figure 4A,B) and that F element probes revealed this same pattern of hybridization in testis (Figure 4C,D). It has been demonstrated that primary spermatocytes are derived from the primary spermatogonial cells by four mitotic divisions that produce 16 primary spermatocytes in the cyst [24].

In the mature egg chamber of the ovaries, which consists of the oocyte and nurse cells that are surrounded by somatically derived follicle cells, we detected sense and antisense transcripts of suffix in the cytoplasm of the nurse cells (Figure 5A,B). The F element probes were also found to hybridize in the cytoplasm of the nurse
cells, but they also reveal the presence of transcripts in the follicle cells (Figure 5C,D). Again, the same patterns were observed for both sense and antisense probes. Although suffix and F element patterns in ovaries have one obvious difference, the patterns revealed by sense and antisense probes for each element were found to be consistent.

It has been postulated that if sense and antisense RNAs are present in the same cell, they can form dsRNA, but it is difficult to directly check for the formation of dsRNA in situ. Our in situ hybridization data from two Drosophila organs indicate that sense and antisense RNAs generated from suffix or F elements are present in the same cells. Hence, there is a potential for the formation of the corresponding dsRNA, at least in some tissues and organs.

**Suffix and RNA silencing mechanisms**

The presence of sense and antisense transcripts of different lengths generated from suffix sequences during all stages of Drosophila development, and the fact that these transcripts might be expressed in the same cells, provides the potential for forming dsRNA species in vivo, and the triggering of RNAi mechanisms leading to sequence-specific degradation of the cognate RNAs [12–14]. To test whether this is the case, we examined the presence of suffix-specific siRNAs in the total RNA samples isolated from embryos, larvae, pupae and imago. Figure 6A shows that siRNAs ranging in length from 21 to 25 nt are observed only in pupae. This result is not due to higher amounts of pupal RNA in the lane, which was tested using 5.8S ribosomal RNA (Figure 6A). These data are also reproducible, as the same results were observed in three experiments with different RNA preparations. Thus, although symmetrical transcription can be observed throughout development, suffix-specific siRNAs are observed during only one particular stage. Of course, we cannot exclude the possibility that smaller quantities of these siRNAs are below the threshold of detection for this method.

To determine whether Dicer is involved in the formation of the detectable 21–25 nt siRNAs, we analyzed total RNA preparations from ovaries of dcr2/dcr2 flies [25]. Using an SI nuclease protection assay, we observed only the longer 26–31 nt small RNAs (Figure 6B). These data clearly demonstrate that Dicer-2 is required for formation of suffix-specific siRNAs. This experiment also drew our attention to the recently detected 24–29 nt small RNAs formed from the transcripts of some retroelements and repetitive elements [20]. These transcripts are not processed into siRNAs in the germ line, but are converted by Dicer-1 and Dicer-2 independent pathways into 24–29 nt rasiRNAs. It is also noteworthy that in the wild type ovaries we detected the formation of both suffix-specific 19–25 nt siRNAs and longer 26–31 rasiRNAs. This indicates that in the ovaries of the Dicer-2 mutant, only one degradation pathway for suffix-containing transcripts is affected, whereas an additional pathway that produces longer RNAs remains active. It follows from these findings therefore that in the germ line, suffix-containing RNAs are controlled by two distinct silencing mechanisms using siRNAs and piRNAs.

**Suffix** is located on the very end of the F element and supplies it with its last conserved RT domain, polyadenylation signal and site [16,18]. For this reason, the degradation of the suffix region in F element mRNA should lead to the silencing of this LINE during the pupal stage of development, at least in some tissues, or in ovaries.

**Analysis of the 3′ ends of F element transcripts**

We employed 3′ RACE to test whether Drosophila pupae contain F element transcripts lacking the suffix region as a result of suffix-
Figure 7A. Poly(A) polymerase was used for the addition of poly(A)$^-$ tail to the poly(A)$^+$ RNA preparations isolated from pupae or ovaries, the samples were used for reverse transcription with a poly(T) primer and subsequent PCR using poly(T)-and F element-specific primers. (B) The number of clones identified by hybridization with suffix-specific or F-element-specific probes in colony-hybridization experiments. Five clones from pupae (3, 5, 16, 28 and 38) showed hybridization with the F-element probe only were selected for sequencing. Similarly, two clones (15 and 55) were isolated from the wild type ovaries. (C) The sequences of the cDNA clones isolated from pupae or ovaries that were truncated by RNA silencing mechanisms at the very beginning of the F-element region (Figure 7B). These data indicate that the levels of 3' truncated F element transcripts are reduced in the Ago2 mutant. Probably the major portion of 3' truncated F element mRNAs comes from RNAi mechanism. Really, the quantitation of the nuclease protection data (line 1, Figure 6C) shows that about 60% of the detected small RNAs correspond to siRNAs. Taken together, these results indicate that in some tissues in pupae and in ovaries, suffix-specific RNA silencing mechanisms are initiated and result in the appearance of 3' truncated F element RNAs lacking suffix sequence possessing the last RT domain. We thus conclude that the observed cut sites in the F element transcripts do not correspond to non-specific degradation, but are produced by RNA silencing mechanisms.

DISCUSSION

We have observed a complex pattern of somewhat long transcripts generated from both strands of suffix and from F elements in Drosophila. Northern blotting and in situ hybridization analysis indicate that suffix transcription is highly complex and is regulated during development. Suffix is mainly transcribed from different sites of insertion as a component of longer RNA molecules, and only a small portion of these correspond to F elements. Separate suffix copies are also much more actively transcribed that those residing in elements. Our data indicate that separate suffix copies are transcribed independently of the F element, but that the transcription of both elements is regulated coordinately. Both elements are expressed in tissues and organs in the same cells, with one exception. Interestingly, both sense and antisense transcripts of both elements are expressed in the same cells.

Both sense and antisense suffix transcripts were found in total RNA preparations during all developmental stages of Drosophila. Nevertheless, our data on siRNA detection and 3'RACE cloning strongly suggest that, at least in some cells in the pupae, suffix-containing RNAs are involved in RNAi mechanisms. We thus conclude that suffix-containing transcripts form dsRNAs that trigger RNAi. Degradation of the 3' end in F element transcripts, where suffix sequences are located, removes part of the coding region, and also the polyadenylation signal and polyadenylation site, and this will necessarily cause silencing of the F element. Suffix is therefore likely to play a role in the regulation of F element expression in some tissues and organs of pupae, which is why we did not observe the degradation of all suffix-containing transcripts isolated from the whole body. We speculate that in some cells suffix serves as a tool for a silencing of the F element.

In previous studies, suffix sequences were found at 3' ends in the ribosomal protein L36A (CG208) and in pOT2 (CG363) genes [16]. In these genes, the suffix regions possess a functional polyadenylation signal and site. It follows therefore that suffix-specific siRNAs could potentially target the corresponding transcripts and give rise to silencing of these genes in some cell types. It is possible also that other genes containing this element in their 5' or 3' non-coding sequences are regulated in the same way.
our present study of 26–31 nt long piRNAs, whereas 19–25 nt long siRNA suffixes have been detected previously in the SINE-containing genes. Concerted silencing of the SINE-containing genes results in the degradation of stretches that are important for gene expression and thus leads to the formation of dsRNA and consequently to RNAi. These data indicate a role of RNAi in gene silencing.

Figure 8. Hypothetical model for the concerted regulation of genes containing a SINE in their 5’ or 3’ non-coding regions. Symmetrical transcription of a SINE sequence, co-transcribed as part of a number of larger RNA molecules, leads to the formation of dsRNA and thus to RNAi. As a result, SINE-specific siRNA molecules target the SINE-containing miRNAs, leading to the cleavage of the 5’ or 3’ non-coding stretches that are important for gene expression and thus result in the concerted silencing of the SINE-containing genes.

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The transcription of both Suffix strands and the degradation of its transcripts by RNA silencing mechanisms are developmentally regulated. The former gives different patterns of element transcription in development, whereas the latter leads to siRNA formation, at least during the pupal stages of development, although Suffix sense and antisense RNAs are even more abundant in embryos and during the imagos stages. It is conceivable that RNAi mechanisms involving these sequences are downregulated in particular organs and tissues during development by as yet unknown factors. In this case, the formation of dsRNA alone is not sufficient to trigger RNAi. Recently, it was shown in D. melanogaster that Dicer-2 is not required for the formation of roo rasiRNA [20]. On the other hand, it was also demonstrated that the overexpression of downstream Argonaute proteins in C. elegans enhances silencing, suggesting that some proteins are limiting for RNAi [27]. It is possible that some tissues and organs in pupae are limited for particular proteins involved in RNAi, and the presence of these proteins are needed for this process to be initiated. Intriguingly, the sizes of the Suffix-specific siRNAs were found to be between 21–25 nt, whereas LTR retroelements, such as roo, med1, and gypsy, non-LTR retroelement and Het-A rasiRNAs are about 24–29 nt long [20]. It has been shown, however, that Dicer-2-dependent siRNAs are produced with a periodicity of 22 nt [28]. Moreover, the analysis of Su(Ste) rasiRNAs has revealed little or no periodicity in processing of its long dsRNA triggers [20]. This may be true also for the suffix-specific RNA silencing mechanisms, as we have observed three excision sites in F element transcripts separated by distances of 4, 6, 9 and 13 nt (Figure 7).

Figure 8. Hypothetical model for the concerted regulation of genes containing a SINE in their 5’ or 3’ non-coding regions. Symmetrical transcription of a SINE sequence, co-transcribed as part of a number of larger RNA molecules, leads to the formation of dsRNA and thus to RNAi. As a result, SINE-specific siRNA molecules target the SINE-containing miRNAs, leading to the cleavage of the 5’ or 3’ non-coding stretches that are important for gene expression and thus result in the concerted silencing of the SINE-containing genes.

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One possible reason for the formation of Suffix-specific siRNAs and piRNAs is that during the pupal stages of D. melanogaster development or in the germline, abnormal suffix-containing transcripts are abolished, and mechanisms that protect against retroelement-containing RNAs are switched on. Another potentially more interesting possibility, however, is that the dispersion of SINEs within transcripts provides a mechanism for concerted gene-silencing. Based on this speculation, SINEs could be considered not as selfish components of the genome, but as elements of biological significance that have important functions at the RNA level as components of RNAi. In this way, the degradation of the regions important for expression of a specific set of genes possessing particular SINE elements in 5’ or 3’ non-coding regions could be achieved (Figure 8).

Suffix has a functional coding sequence that is part of the F element at the protein level. Moreover, it functions also at the RNA level, since it is involved in RNA-regulation. The protein domain specified by Suffix within the F element forms an 8th conserved domain of the reverse transcriptase [31]. The 7 conserved domains have been described for different retroelements [32]. Some specific groups of LINEs, including F elements, have additional ancient RT domains [Kretova and Tchurikov, unpublished]. Evolutionary pressure on Suffix could act upon both the RT and RNA-regulation functions of the element. Both mechanisms—reverse transcription and RNAi—are considered...
ancient and evolutionary conserved mechanisms for the synthesis of DNA copies on RNA templates, and for the regulation of the expression of host or foreign RNAs. The function of suffix as a conserved SINE remains unknown however and as a separate element, suffix is clearly unable to serve as a coding sequence. There are some data on the role of SINES in stress defense and in the post-transcriptional stimulation of expression of different mRNAs [2,3,34]. Our present experiments with heat-shock treated flies did not detect any change in the suffix transcription pattern (data not shown).

Separate suffix copies could originate from the 3' end of the F element. Recently, we identified a weak internal promoter associated with retroposition. In return, suffix may downregulate F elements by RNA silencing mechanisms, which probably allow this LINE/SINE family to replicate without killing the host.

MATERIALS AND METHODS

Northern-blot analysis

Approximately 20 μg of poly(A)⁺ or poly(A)⁻ RNA samples isolated from the Oregon-Shostak line were electrophoresed in 2 mm thick, 1.2% agarose gels containing 25 mM NaPO₄ (pH 7), 0.5 mM EDTA and 5% formaldehyde and blotted in 20×SSC onto Hybond-N+. Hybridization was performed in a solution containing 50% formamide, 5×SSC, ficoll, polyvinylpyrrolidone, BSA, and SDS all at a concentration of 0.1%, denatured salmon DNA (50 μg/ml), tRNA (50 μg/ml) and 5-10×10⁵ cpm of the appropriate probe. 10⁵ cpm/μg of the corresponding RNA probes were synthesized with T7 RNA polymerase in vitro using adjacent fragments of F element-containing clones as shown in Figure 1. These corresponding fragments had been subcloned into either pGEM-1 or pGEM-2 vectors. The final plasmids were linearized with a restriction enzyme appropriate probe. 10⁹ cpm/?l of the RNA probe. After hybridization, 2 μl aliquots were mixed with 10 μl of solution containing 50 mM sodium acetate (pH 4.5), 0.28 M NaCl, 4.5 mM ZnSO₄ and 0.1–0.8 μl of SI nuclease (Promega). SI digestion was performed for 30 min at 20°C, followed by the addition of a 10 μl solution containing 90% formamide, 20 mM EDTA and dyes. The probes were separated in 12% denaturing, 0.2 mm, polyacrylamide gels.

The detection of small RNAs via an SI nuclease protection assay

Strand-specific DIG-labeled RNA probes were transcribed by T7 RNA polymerase. About 1 μg of DNA template was used in a 20 μl transcription reaction mixture as described above, but now containing ATP, CTP, GTP, CTP (500 mM each), 1.25 μM [α-³²P] UTP (6000 Ci/mmol, EIMB), 10 μM unlabelled UTP and 20 u of T7 RNA polymerase (Fermentas). The RNA was gel purified to remove shorter fragments. About 5 μg of the total Drosophila RNA preparation was hybridized at 50°C for 12 h in 20 μl of solution containing 0.7 M NaCl, 0.1 M Tris-HCl (pH 7.4), 0.1% SDS and 10⁵ cpm of the RNA probe. After hybridization, 2 μl aliquots were mixed with 10 μl of solution containing 50 mM sodium acetate (pH 4.5), 0.28 M NaCl, 4.5 mM ZnSO₄ and 0.1–0.8 μl of SI nuclease (Promega). SI digestion was performed for 30 min at 20°C, followed by the addition of a 10 μl solution containing 90% formamide, 20 mM EDTA and dyes. The probes were separated in 12% denaturing, 0.2 mm, polyacrylamide gels.
blocking and in the same solution with anti-DIG-alkaline phosphatase antibodies (Roche, 1:2000) for 1 h. Finally, samples were washed five times for 15 min in the blocking solution and once for 15 min in PBT. For staining reactions, samples were washed for 10 min in alkaline phosphatase buffer, containing 0.1 M NaCl, 50 mM MgCl₂, 100 mM Tris, pH 9.5, 0.1% Tween 20, and incubated with 1 ml of the buffer containing 20 µl of nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) stock solution (Roche). Development of the reaction was observed visually under the microscope, and the reaction was usually stopped after 0.5 to 1 h. Samples were then washed five times for 3 min with PBT and mounted in 60% glycerol in PBS.

3’RACE

For the cloning of 3’ ends of F element transcripts lacking suffix and poly(A) sequences, the addition of poly(A) stretches was performed with the aid of yeast poly(A) polymerase (see the scheme in Figure 7). 10 µg of total poly(A) RNA isolated from pupae or from ovaries was incubated for 10 min at 30°C in a buffer containing 25 mM Tris-HCl (pH 7.0), 40 mM KCl, 0.5 mM MnCl₂, 0.05 mM EDTA, 0.5 mM DTT, 0.2 mg/ml BSA, 10% glycerol, 3.3 µM [α-32P]ATP (6000 Ci/mmol, EMBI), 0.5 mM ATP and poly(A) polymerase (USB). After ethanol precipitation, the sample was used for reverse transcription with an oligo(dT)20 primer. This was followed by a 10 cycle PCR amplification in a solution containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2 mM MgCl₂; 0.01% gelatin w/v; 1 mM dNTPs; 1 µg of oligo(dT)20 primer; 1 µg of a specific primer with an artificial EcoRI site; 1 µ of Taq polymerase and 1 µ of Tth polymerase. Amplification conditions were 90°C melting, 37°C annealing and 72°C for extension, for 1 min each. The specific primer, 5’ GAGCACAATCAAGATTCTGAGAACCATCA 3’, corresponds to the region located about 120 bp upstream of suffix in the F element. The cloning was performed using an EcoRI-SmaI digested pUC12 vector. For colony hybridization, an F element specific oligonucleotide, corresponding to the region located about 80 bp upstream of suffix, and suffix-specific probes were used. The clones hybridizing only with the F element probe were selected and sequenced.

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Author Contributions

Conceived and designed the experiments: NT OK. Performed the experiments: NT OK. Analyzed the data: NT OK. Contributed reagents/materials/analysis tools: NT OK. Wrote the paper: NT OK.

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