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Gag proteins of the two *Drosophila* telomeric retrotransposons are targeted to chromosome ends

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Drosophila telomeres are formed by two non-LTR retrotransposons, *HeT-A* and *TART,* which transpose only to chromosome ends. Successive transpositions of these telomeric elements yield arrays that are functionally equivalent to the arrays generated by telomerase in other organisms. In contrast, other *Drosophila* non-LTR retrotransposons transpose widely through gene-rich regions, but not to ends. The two telomeric elements encode very similar Gag proteins, suggesting that Gag may be involved in their unique targeting to chromosome ends. To test the intrinsic potential of these Gag proteins for targeting, we tagged the coding sequences with sequence of GFP and expressed the constructs in transiently transfected *Drosophila*-cultured cells. Gag proteins from both elements are efficiently transported into the nucleus where the protein from one element, *HeT-A,* forms structures associated with chromosome ends in interphase nuclei. Gag from the second element, *TART,* moves into telomere-associated structures only when co-expressed with *HeT-A* Gag. The results suggest that these Gag proteins are capable of delivering the retrotransposons to telomeres, although *TART* requires assistance from *HeT-A*. They also imply a symbiotic relationship between the two elements, with *HeT-A* Gag directing the telomere-specific targeting of the elements, whereas *TART* provides reverse transcriptase for transposition.

Introduction

HeT-A and *TART*, the two retrotransposable elements that make up telomeres in *Drosophila*, are a bona fide part of the cellular machinery, but they also have features that characterize other transposable elements (Fig. 1). Their most obvious difference from other *Drosophila* non-LTR retrotransposons is their specific transposition to form arrays at the extreme ends of the chromosomes (for reviews see Pardue and DeBaryshe, 1999, 2002).

HeT-A is several times more abundant than *TART*; however, the two elements appear to be more or less randomly mixed in the telomere arrays. Both are non-LTR retrotransposons, and their invariant polar orientation on chromosome ends is explained by the mechanism that this class of elements uses for transposition: the 3′ end of the RNA transposition intermediate is aligned with the target site and reverse transcribed directly onto the chromosome. For most non-LTR elements, the reverse transcription is primed by a 3′ hydroxyl exposed at a nick in chromosomal DNA (Luan et al., 1993; Eickbush, 2002). Reverse transcription of *HeT-A* and *TART* is hypothesized to be primed by the 3′ hydroxyl on the extreme end of the chromosome (Biessmann et al., 1992; Levis et al., 1993).

In addition to *HeT-A* and *TART*, which transpose only to telomeres, *Drosophila* contains other non-LTR retrotransposons that transpose into many parts of the genome, but not into *HeT-A*/*TART* telomere arrays. With a few exceptions, transposition of non-LTR elements does not appear to be targeted by specific DNA sequences at the insertion site. For example, *HeT-A* and *TART* have been found joined to many different sequences in "healing" broken chromosome ends (Biessmann et al., 1990, 1992; Sheen and Levis, 1994; Golubovsky et al., 2001). The lack of specific nucleotide sequence targets suggests that the targeting of the telomere elements may be governed by proteins associated with chromosome ends. These same proteins might serve to exclude nontelomeric elements from the terminal arrays.

The apparently random mixture of *HeT-A* and *TART* in telomere arrays suggests that the two elements have equivalent roles at the chromosome end. However, none of the *D. melanogaster* stocks studied have completely lost either element. The results presented here support the hypothesis that the two elements have a symbiotic relationship, with both elements contributing to their telomere-specific transposition.

Despite their role in forming telomeres, *HeT-A* and *TART* share characteristics of other retrotransposons. For example,

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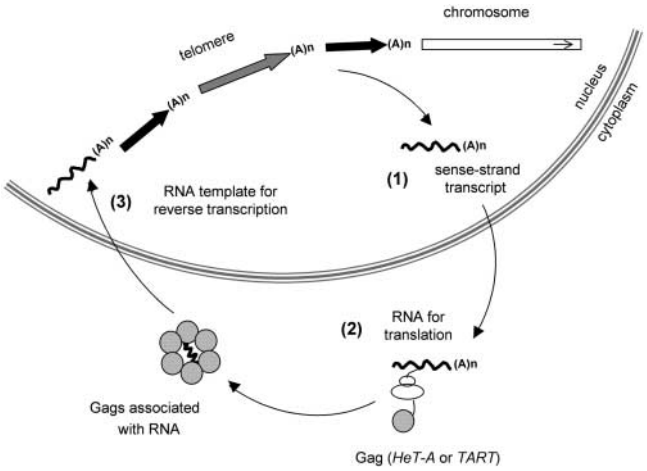

Figure 1. **Model for extension of chromosome ends by telomeric retrotransposons.** Retrotransposons yield a sense-strand transcript that serves as both mRNA and transposition intermediate. This diagram shows our current model for the path of this RNA from transcription until it is reverse transcribed to add another repeat onto the telomere array. Gray arrows represent *HeT-A* (black) and *TART* (gray) elements attached to the end of the chromosome. A poly(A)$^+$ sense–strand RNA is transcribed from a member of the array (1). For the telomeric retrotransposons, there is evidence suggesting that this RNA must be translated (2) before serving as a template (3) for telomere additions (for review see Pardue and DeBaryshe, 2002). This suggestion is now supported by our findings (described in this report) that translation products (Gags) of these RNAs appear capable of delivering the transposition template specifically to its target at the telomere. Gray circles in diagram represent Gags of either *HeT-A* or *TART*. Our results show that *TART* Gag targeting to telomeres depends on its interaction with *HeT-A* Gag. Although the cytological site where the two proteins first associate has not been determined, we have preliminary evidence that this association occurs in the cytoplasm, possibly with the formation of the Gag–RNA complex (unpublished data). Analogy with retroviruses suggests that reverse transcriptase is also included in this complex; however, we have no evidence on this point.

TART has both the *gag* and *pol* coding regions typical of many retrotransposons. The *pol* region encodes reverse transcriptase. The sequence of this enzyme has been used to deduce phylogenetic relationships of retroelements. The analysis places *TART* into the *jockey* clade of insect non-LTR retrotransposons (Malik et al., 1999).

Surprisingly, *HeT-A* does not have a *pol* coding region and must obtain its reverse transcriptase activity from some other source. Whatever the source of this activity, *HeT-A* has been found to transpose much more frequently than *TART* (Savitsky et al., 2002). It is possible that *TART* provides the reverse transcriptase for *HeT-A*, but at this time, there is no evidence to support this suggestion. If *TART* does provide this activity for transposition, it raises the question of why *HeT-A* is more abundant than *TART*. Is *HeT-A* also supplying a necessary function?

In addition to their unique ability to transpose only to chromosome ends, *HeT-A* and *TART* also encode closely related Gag proteins (Pardue et al., 1996; Rashkova et al., 2002). This suggested that the Gag proteins might be involved in the telomere targeting, a suggestion supported by what is known of retroviral Gags, which are responsible for forming ribonucleoprotein particles that carry viral RNA through the cell. For example, retroviral Gag protein has been shown to be both necessary and sufficient to form a capsid localized to the appropriate region of the cell plasma membrane (for review see Swanstrom and Wills, 1997). Here, we explore a possibly analogous role for the Gag proteins of *HeT-A* and *TART* in positioning these elements at telomeres.

The hypothesis that Gag proteins have a role in the telomeric localization of *HeT-A* and *TART* is supported by evidence that the intracellular localization of these Gag proteins is significantly different from that of Gags of non-LTR elements that transpose only to nontelomeric sites in *D. melanogaster* chromosomes (Rashkova et al., 2002). The comparisons were performed by cytological localization of each protein in transiently transfected cultured *Drosophila* cells. Each Gag coding region was tagged with GFP. All proteins were expressed from the same promoter construct so that localization would be determined only by protein sequence, rather than by secondary factors such as promoter strength. The two telomeric transposon Gags were transported rapidly and efficiently into the nucleus. Gags of the nontelomeric retrotransposons had a very different localization. For two elements (*Doc* and *I* factor), essentially all of the proteins remained in the cytoplasm, whereas for the third element (*jockey*), only a small fraction reached the nucleus.

The efficient nuclear localization of *HeT-A* and *TART* Gags is consistent with the status of these elements as part of the cellular machinery (maintaining the chromosome ends) while the presumably parasitic elements are impeded in travel to the nucleus. The unexpected finding was that, inside the nucleus, *HeT-A* Gag and *TART* Gag had very different distributions. This raises the question of how their localization relates to the final transposition of these elements to telomeres. We now report further studies showing that *HeT-A* Gag is preferentially associated with chromosome ends. *TART* Gag does not associate with telomeres unless the two proteins are coexpressed. In such cells, *HeT-A* Gag efficiently redirects *TART* Gag to telomeres.

Results and discussion

Gags from *HeT-A* and *TART* have different nuclear localizations

As soon as these proteins are detected in transfected cells, they are found almost entirely within the nucleus (Rashkova et al., 2002). *HeT-A* Gag forms many tiny dots, which then aggregate into larger, fairly regular structures that we call Het-dots. These dots are distributed through the nucleus with a tendency to be found along the nuclear membrane (Fig. 2 A). *TART* Gag has a more diffuse distribution, forming irregular small clusters associated with less condensed material (Fig. 2 B).

When cells are examined after being simply dropped on a slide, the nucleus is thick enough that it is necessary to use optical sectioning to visualize all the Het-dots. The cells used in this study, Schneider line 2 (SL2),* have a diploid karyotype with a single X chromosome and three pairs of autosomes. The population is a mixture of diploid and tetra-

*Abbreviation used in this paper: SL2, Schneider line 2.

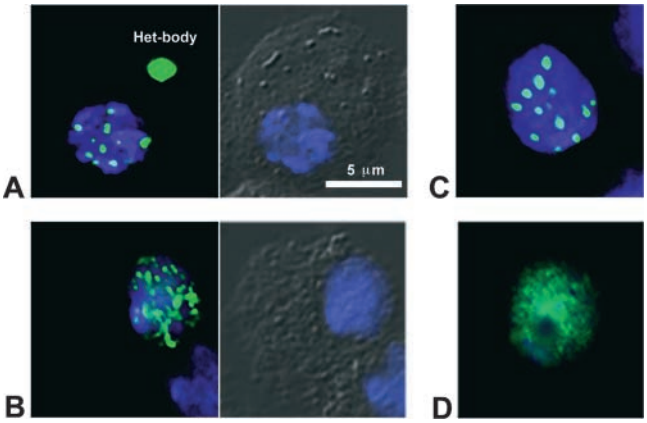

Figure 2. **Intracellular localization of *HeT-A* Gag and *TART* Gag in interphase cells.** Fluorescence micrographs of SL2 cells transiently transfected with constructs encoding *HeT-A* and *TART* Gags fused with GFP. DNA stained with DAPI (blue). Cells in A and B were dropped on slides. (left) Merged GFP and DAPI images. (center) DAPI and DIC. Cells in C and D were centrifuged. (right) Merged GFP and DAPI. (A) *HeT-A* Gag forms Het-dots in the nucleus and Het-body in the cytoplasm. Het-dots appear to be different sizes because the micrograph is an optical section of nucleus. (B) *TART* Gag forms small clusters spread through nucleus. (C) *HeT-A* Gag in Het-dots withstands centrifugation. (D) *TART* Gag clusters do not survive centrifugation; instead, protein spreads through the nucleus, avoiding the nucleolus.

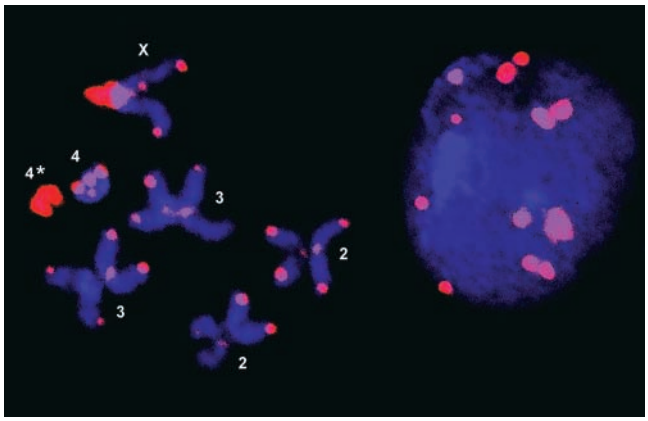

Figure 3. **Localization of HOAP in metaphase and interphase cells.** Two adjacent SL2 cells stained with anti-HOAP serum and Cy3-secondary antibody (red) and DAPI (blue). In metaphase (left cell) HOAP stains dots at tips on both chromatids of each of the seven chromosomes (identified on figure), and shows faint stain in pericentric regions of autosomes. Asterisk marks smaller chromosome 4. In interphase nuclei (right cell) HOAP forms relatively uniform dots approximately equal to the number of telomeres.

ploid cells, so nuclei should have 14 or 28 telomeres before chromosomes are replicated. These numbers give limits of the number of Het-dots expected if Het-dots are completely associated with telomeres. However, the number might appear to be reduced by telomere fusions or overlaps in these tiny nuclei. In addition, dots may not associate with every telomere in any given nucleus. We find that the numbers of dots vary, but in many nuclei there are 10–14 dots.

To deposit the Het-dots in a single plane and spread them over a larger area, we have used a cytocentrifuge to flatten or break the nuclei. These preparations show the same numbers of Het-dots as seen by optical sections of dropped cells. However, the centrifuged cells reveal a clear difference in stability between Het-dots and *TART* Gag clusters (Fig. 2, C and D). Het-dots withstand spinning, whereas *TART* Gag clusters break down and the protein spreads through the nucleus. Apparently, protein associations in *TART* Gag clusters are not strong enough to withstand centrifugation.

In addition to differences in stability between Het-dots and *TART* Gag clusters, there is one unusual aspect of *HeT-A* Gag localization never seen with *TART* Gag or other retrotransposon Gags. About a third of the cells with Het-dots have a large smooth-edged body of cytoplasmic *HeT-A* Gag protein, usually well removed from the nucleus. We refer to this structure as the Het-body (Fig. 2 A). It is never seen in cells that do not have nuclear *HeT-A* protein. Cells with Het-bodies can still divide; we have observed them in telophase. In these cells, there was only a single Het-body and it is not clear how the material is eventually distributed between the daughters. We assume these bodies reflect overexpression of Gag, but if so, this overexpression shows that the cells treat this protein differently from other excess Gag protein. Other presumably overexpressed proteins form multiple aggregates, associated with more diffuse material, broadly distributed over the cytoplasm.

Het-dots are preferentially associated with chromosome ends

The number and localization of Het-dots fit the expectation for structures associated with chromosome ends. To test this hypothesis we looked for, but did not find, association of the dots with metaphase chromosomes. Instead, we found that both *HeT-A* and *TART* Gags diffuse throughout the cell at metaphase (Rashkova et al., 2002) with a few streaks of aggregated protein remaining. This behavior is similar to that reported for the chromatin protein, HP1, in *Drosophila* (Kellum et al., 1995) and for several sequence-specific transcription factors in human cells (Martinez-Balbas et al., 1995). Nuclear associations of *HeT-A* and *TART* Gags appear to reform during telophase; thus, testing the relation of Het-dots to telomeres requires a marker that can identify chromosome ends in interphase nuclei.

A number of telomere-associated proteins have been characterized for mammals and for yeast, but *Drosophila* telomere–associated proteins are still relatively unknown. One protein, HOAP, has been shown to associate predominantly with telomeres in *Drosophila* polytene chromosomes (Shareef et al., 2001). Polytene nuclei are interphase nuclei; however, we now find that HOAP remains on the chromosome throughout the cell cycle and can be detected on metaphase chromosomes in the cultured cells. The major sites of anti-HOAP antibody binding are the telomeres (Fig. 3). HOAP is found on all telomeres, although some chromosome ends stain less heavily than others. The relative staining level is similar on sister chromatids, suggesting that the amount of HOAP present may be characteristic of specific ends.

A striking feature of each metaphase spread in the cultured cells is a bright doublet of HOAP staining, seen once in diploid spreads and twice in tetraploid spreads. The DAPI image reveals that this heavily stained body is one of the tiny fourth

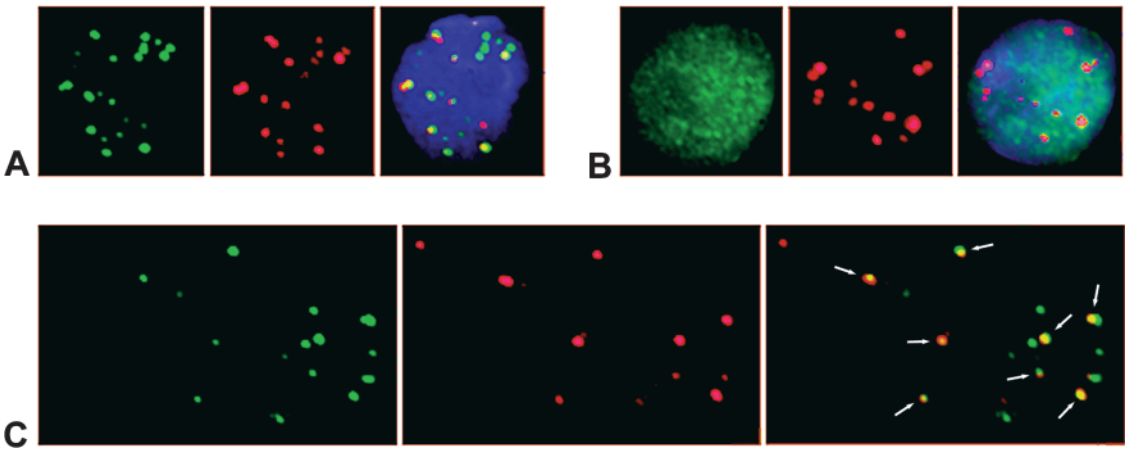

Figure 4. **Colocalization of Het-dots and HOAP dots in centrifuged cells and broken spread nuclei.** Interphase SL2 cells transfected with either *HeT-A* Gag or *TART* Gag and stained for HOAP (red) and DAPI (blue). GFP-tagged Gag and HOAP images are shown separately as well as superimposed (third panel). (A) *HeT-A* Gag. In the nucleus of a centrifuged cell, the majority of Het-dots coincide with HOAP dots. (B) *TART* Gag. In the nucleus of a centrifuged cell, *TART* Gag protein is dispersed through the nucleus around the nucleolus. (C) *HeT-A* Gag in broken, spread nucleus, showing that the association of HeT-dots and HOAP dots is maintained when nuclear contents are widely spread (micrograph at same magnification as A and B). Coinciding dots are marked with arrows. DNA is uniformly spread over the nucleus and DAPI omitted for clarity.

chromosomes and is somewhat smaller than its homologue. It appears that one chromosome 4 in our cell line has lost part of its sequence, but this loss has not reduced the amount of HOAP on the chromosome. In addition, we see faint HOAP staining at the centromeres of the large autosomes. Centromere staining also may be present on the X and fourth chromosomes, but would not be differentiated from the nearby telomere of the short arm of either of these chromosomes. In interphase nuclei, HOAP staining appears as dots whose number is in the range expected for telomeres in these cells.

These studies of metaphase chromosomes show that HOAP staining serves as a marker for chromosome ends through the cell cycle. Thus, the relation between Het-dots and telomeres in interphase nuclei can be analyzed by comparing the distribution of Het-dots with that of HOAP dots. To minimize nonspecific overlap in these small nuclei, we have done the analyses on centrifuge-flattened cells and broken nuclei. We find many of the Het-dots, ranging from 60 to 90% for different cells, overlap with HOAP dots. On visual inspection of spread nuclei, it is clear that Het-dots and HOAP dots associate closely (Fig. 4, A and C).

We conclude that there is preferential association of Het-dots with chromosome ends, and this association is strong enough to withstand centrifugation. Although HOAP identifies chromosome ends, it may not be directly associated with Het-dots. We note that some of the Het- and HOAP dots, though clearly associated, do not entirely overlap (Fig. 4 C). The partial overlaps could be due to instrumentation bias in aligning the images, but because the misalignment is in different directions for different spots, it seems likely the Het- and HOAP dots are associated with slightly different parts of the chromosome end, and therefore settle on the slide in different orientations.

Even if the two proteins were directly complexed at telomeres, one should not necessarily expect HOAP and Het-dots to be completely associated. Some telomeres might not have associated Het-dots because there is no reason to suppose that every telomere should have associated Het-dots in any given cell cycle. In addition, HOAP at centromeres should not have corresponding Het-dots. Furthermore, sample preparation could disrupt some associations between Gag, HOAP, and/or other components of the complexes at the chromosome ends.

HeT-A Gag recruits *TART* Gag to specific locations

TART Gag does not associate preferentially with chromosome ends. We see no significant coincidence between *TART* Gag and HOAP in nuclei that have not been centrifuged. The association cannot be studied in centrifuged cells because the *TART* Gag clusters break down and the protein is spread over most of the nucleus around the nucleolus (Fig. 4 B).

This distribution of *TART* Gag changes dramatically when the protein is coexpressed with *HeT-A* Gag. For these experiments, *TART* Gag was tagged with YFP and *HeT-A* Gag with CFP because these two fluorochromes can be detected separately in the same preparation. In single transfections, YFP- and CFP-tagged Gag proteins behaved exactly as did their GFP-tagged counterparts.

When *HeT-A* Gag is coexpressed with *TART* Gag, the two proteins colocalize completely (Fig. 5 A). The localization is controlled by *HeT-A* Gag. *TART* Gag is seen in Het-dots and also in Het-bodies. The association between the two proteins is strong enough to withstand centrifugation. Preliminary experiments with deletion derivatives of the proteins (unpublished data) have shown that the association is dependent on amino acid sequences in the region of the zinc knuckles of both proteins (Pardue et al., 1996).

The *HeT-A–TART* Gag colocalization is specific; other closely related Gags are not recruited

The observation that *HeT-A* Gag completely dominates and redirects the localization of *TART* Gag when the two proteins are coexpressed raised the question of whether this interaction is specific for the two telomere Gags. Sequence analyses show

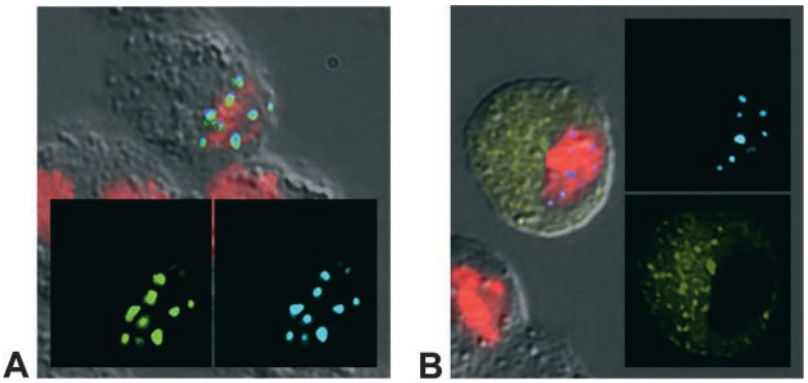

Figure 5. ***HeT-A* Gag affects the localization of *TART* Gag, but not *I* factor Gag, in interphase nuclei.** Co-transfected cells expressing CFP- and YFP-tagged proteins. Panels show merged YFP, CFP, DAPI (false-colored red), and DIC. Insets show CFP and YFP alone. (A) *HeT-A* Gag (CFP) and *TART* Gag (YFP). Both proteins localize to Het-dots. (B) *HeT-A* Gag (CFP) and *I* factor Gag (YFP). *HeT-A* Gag forms Het-dots, but *I* factor Gag remains in the cytoplasm.

that *TART* Gag is very closely related to *HeT-A* Gag. However, Gag proteins from some of the nontelomeric insect retrotransposons also show good similarity to *HeT-A* Gag (Pardue et al., 1996). We have tested the possibility that three of these proteins, *Doc* Gag, *jockey* Gag, and *I* factor Gag, might also colocalize with *HeT-A* Gag. These experiments were performed by the protocol used for studying coexpression with *TART* Gag. None of the Gag proteins from these nontelomeric elements had its localization affected by coexpression with *HeT-A* Gag (Fig. 5 B). We conclude that the colocalization of *HeT-A* and *TART* Gags is not a generalized Gag–Gag interaction, but that it shows strong specificity. This specificity appears to be biologically relevant because these proteins are encoded by elements with the same transposition targets.

Conclusions

Drosophila is remarkable for adapting two non-LTR retroelements to maintain its telomere arrays (Fig. 1). In this paper, we show that Gag proteins encoded by these elements have the potential to target their transposition intermediates to chromosome ends. Our finding that *HeT-A* Gag overrides the localization of *TART* Gag in cotransfections leads to an intriguing speculation about the roles of each of these elements in forming *Drosophila* telomeres. *HeT-A* does not encode a reverse transcriptase but *TART* does. *TART* may provide this activity for both elements, whereas *HeT-A* may be responsible for the final targeting of both retrotransposons to the telomere. This role in targeting can explain why *HeT-A*, the element lacking its own reverse transcriptase, is so abundant. The colocalization suggests that these two telomeric transposons may have coevolved into symbiotes, with *TART* supplying the reverse transcriptase and *HeT-A* the nuclear targeting.

Like other metazoa, *Drosophila* has many kilobases of DNA in its telomere arrays. Little is known about rates of turnover and replacement on normal telomeres in metazoa; however, studies on yeasts and other organisms reveal that telomere sequences are in dynamic flux, with sequence gains and losses that are influenced by genetic background, by growth conditions, by cell type, and by developmental stage (Blackburn, 2001). The experiments described here study the behavior of overexpressed proteins, but they reveal a mechanism of retrotransposon localization that has the flexibility to maintain the dynamic telomeres suggested by the yeast studies. This system is efficient; almost nothing is left behind, arguing that even a small amount of expressed Gag protein would get to a telomere. This system is also robust because it can accommodate a significant amount of protein before formation of the cytoplasmic Het-body, which appears to represent an overload of the system. Such a mechanism has the capacity to respond rapidly to the need to change telomere length; an important adaptive mechanism for the cell.

Materials and methods

Recombinant DNA and plasmid construction

The Gag–GFP constructs have been described previously (Rashkova et al., 2002). Each coding sequence was fused to sequence for EGFP in pPL17. For coexpression, sequences were recloned in pSR24 and pSR25, respectively, expressing ECFP and EYFP under the armadillo promoter. They were constructed by inserting the BamHI-StuI fragments from vectors pECFP-N1 and pEYFP-N1 (CLONTECH Laboratories, Inc.) into pPL17 cut with BamHI and partially cut with StuI, thus replacing the coding sequence of EGFP with ECFP or EYFP.

Cell culture and transfection

Drosophila SL2 cells were maintained in DME supplemented with 10% FCS, 0.5% lactalbumin hydrolysate, and 10 mM nonessential amino acids. For transfection, 2.5×10^6 cells/ml in 5 ml of DME were incubated at 25°C for 18–20 h. Transfections were performed in 2.5 ml of serum-free DME using a Cytofectene Transfection Reagent Kit (Bio-Rad Laboratories) and 5–10 μg of plasmid DNA purified with a Plasmid Midi Kit (QIAGEN). Medium containing DNA was replaced after 6 h with DME plus 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were analyzed at 24 and 48 h.

Slide preparation

Transfected SL2 cells were dropped onto slides 48 h after transfection and allowed to settle for 20 min, or diluted 10-fold with 1×PBS and spun onto slides for 3 min in a cytocentrifuge at 1,600 rpm. Cells were fixed with 3.7% formaldehyde in PBT (1×PBS; 0.1% Tween 20) for 30 min, washed three times for 5 min in PBT, and stained with 0.2 μg/ml DAPI in 20 mM Tris-HCl, pH 7.4, for 1 min. Slides were mounted in 1×PBS, 50% glycerol. For anti-HOAP staining, nontransfected cells were treated with 5 μg/ml colchicine for 3 h, diluted 10-fold with 0.5% sodium citrate for 5 min, spun onto slides in a cytocentrifuge, and fixed as described above. Slides were incubated 30 min at RT with blocking solution (10% FCS in PBT) for 2 h with a 1:2,000 dilution of rabbit anti-HOAP antibody (Shareef et al., 2001) with Cy3-secondary antibody (Jackson ImmunoResearch Laboratories), and then stained with DAPI.

Microscopy

Fluorescence miscroscopy used an Eclipse microscope (model E600; Nikon) equipped with a CCD camera. Images were taken using Spot RT v3.0 software and processed with Adobe Photoshop® 5.5.

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