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Telomerase-independent mechanisms of telomere elongation

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Abstract. The ends of linear chromosomes must be elongated in a DNA-replication-independent fashion. For chromosome end elongation the majority of eukaryotes use a specialized reverse transcriptase, telomerase, which adds a short, tandemly repeated DNA sequence motif to chromosome ends. Chromosome elongation can also be achieved, however, by mechanisms other than telomerase. Such elongation events have been detected under conditions where telomerase has been inactivated experimentally and in the few organisms that naturally lack telomerase. We will summarize current knowledge on these telomerase-independent elongation mechanisms in yeast and mammalian cells and will discuss in more detail the telomere elongation mechanism by retrotransposons in *Drosophila melanogaster*.

Key words. Telomere; recombination; gene conversion; transposable elements; capping; *Drosophila.*

Experimentally generated uncapped chromosome ends

The ends of linear chromosomes, called telomeres, are extended in most eukaryotes by the action of telomerase, a reverse transcriptase that copies short repeats from a specific RNA template. But chromosome end elongation can also be achieved by telomerase-independent mechanisms when telomerase has been inactivated in organisms that normally use telomerase or in the few organisms that naturally lack telomerase. As excellent reviews on non-telomerase-mediated telomere elongation in yeast and mammalian cells have appeared recently [1–4], we will emphasize the telomere elongation mechanism by retrotransposons in *Drosophila melanogaster*.

Some 60 years ago H. J. Muller irradiated *Drosophila* males and followed the fate of double strand breaks (DSBs) produced in germ cells. He found a variety of rearranged chromosomes in the offspring, but terminally deficient chromosomes were never recovered. These results led him to define the telomere and postulate its capping function [5, 6], which is to distinguish natural chromosome ends from DSBs. Following broken chromosomes that originated from dicentrics during meiosis of *Zea mays*, B. McClintock reached similar conclusions [7, 8]. Uncapped chromosome ends without telomeric repeats can be generated experimentally; methods include irradiation, breaking dicentric chromosomes and cutting genomic DNA with site-specific endonucleases. Studying the fate of these abnormal chromosome ends in yeast has revealed important mechanisms of chromosome healing [9–13]. Uncapped ends trigger a cell cycle checkpoint-mediated arrest, but can acquire a new telomere by de-novo initiation of telomerase at the break, or more often by capture of a telomere using RAD52-dependent break-induced recombination, BIR [14]. BIR is a nonreciprocal replication process that is also the basis of gene conversion and gene targeting. This process is initiated when a broken chromosome end is resected by endonucleases to generate a 3′ overhang, which can undergo homology-dependent strand invasion to initiate replication of the invaded DNA duplex (fig. 1). When a DSB in a chromosome arm shares short sequence homology with a region near another chromosome end, telomere capture events can occur that will restore a new telomere at the broken end [12].

Uncapped chromosomes may also occur after complete or partial inactivation of telomerase or crucial accessory proteins at the telomere [15–18], which leads to telomere shortening. Telomere repeat loss beyond a certain thresh-

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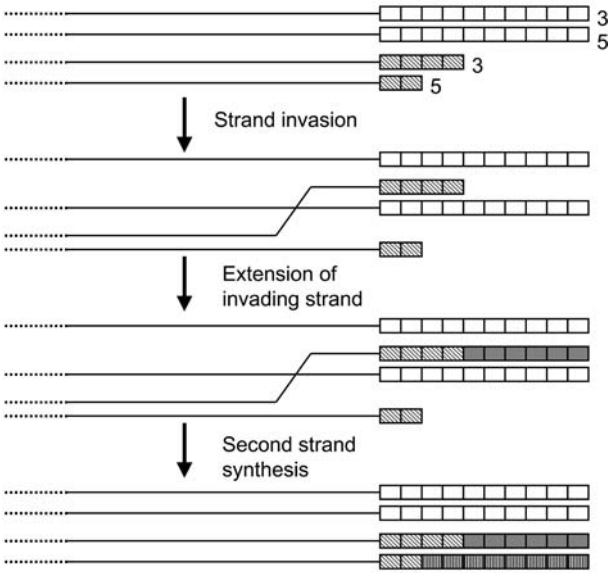

Figure 1. Model of terminal conversion triggered by invasion of the telomeric repeat region of the homologue by the 3′ overhang and subsequent extension of the invading strand by DNA replication. Telomeric repeats are depicted as rectangles. Unfilled and diagonally hatched rectangles distinguish telomeric repeats on different chromosomes. Gray-shaded rectangles symbolize repeats copied from the invaded telomeric repeat region, and vertically hatched rectangles are repeats synthesized by normal DNA synthesis.

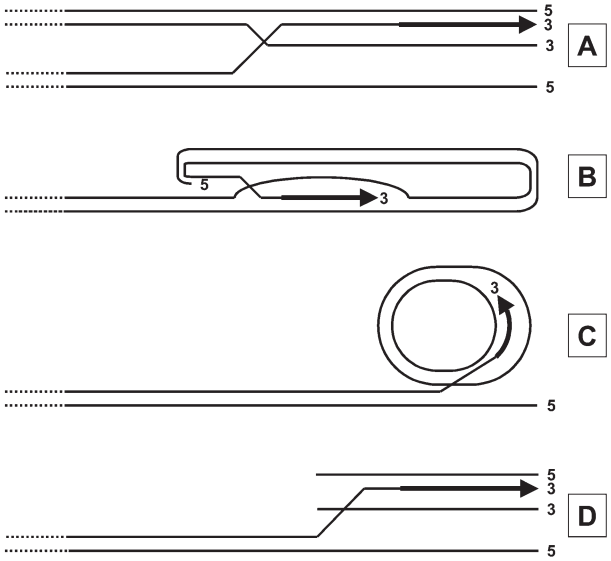

Figure 2. Possible choices of different templates for BIR. (*A*) intermolecular homologous telomere, (*B*) intramolecular invasion forming a d-loop on the same chromosome, (*C*) extrachromosomal circular and (*D*) small linear DNA. The arrows indicate direction of 3′ extension of the invading strand.

old will eventually lead to an altered telomere structure, disturbing the binding of telomeric proteins such that the telomere's capping function is compromised, and the chromosome end becomes recognized as a DSB. The resulting phenotypes, such as chromosome end-to-end fusions [19] and increased mutation rate, probably caused by bridge-breakage-fusion cycles [20], support this notion.

In *Saccharomyces cerevisiae*, not all cells with dysfunctional telomeres die. Survivors arise that appear to have restored a functional telomere by using a *RAD52*-dependent recombination mechanism [21, 22]. Survivors classified as type I show drastically amplified Y DNA elements that are found in the subtelomeric region of most chromosomes and retain very short terminal repeats. Type-II survivors have long, heterogeneous telomere tracts. In both cases, repeat sequences are extended at the chromosome ends, restoring at least some telomeric capping function, which result in a growth advantage. Even though telomeres continually shorten in type-II survivors, growth eventually stabilizes, suggesting that recombination alone is sufficient to maintain a functional telomere. The BIR mechanism may be responsible for the higher number of tandem repeats at chromosome ends in these survivors. The single strand of a shortened telomere may invade the telomere of another chromosome (fig. 2A) or proximal homologous sequences on the same chromosome arm generating a DNA loop (fig. 2B). Extrachromosomal circular (fig. 2C) or linear molecules (fig. 2D) may also be used as replication templates. A rolling-circle model is supported by observations of type-II survivors [23], where abrupt size increases of telomere repeat tracts were larger than expected if the other telomeres had been used as templates, and these rapid size increases occurred only at critically short telomeres. This saltatory behavior of telomere tract extension is reminiscent of the abrupt shortening of elongated telomeric tracts [24, 25] and suggests a common mechanism such as intrachromatid recombination with an intermediate structure, shown in figure 2B, in which the single-stranded terminal 3′ end invades proximal telomeric repeats. Through branch migration, nicking in the d-loop and resolution of the Holliday junction, large DNA pieces can be excised, resulting in rapid telomeric deletion. On the other hand, the invading strand can be extended by DNA replication, giving rise to the rapid telomere extensions.

In *Kluyveromyces lactis*, loss of telomerase function also leads to telomere shortening, senescence and *RAD52*-dependent type-II survivors, but the mechanism by which these survivors maintain their telomeres seems to differ from that for *S. cerevisiae*. A URA3 marker inserted into a subtelomeric region could either be lost or spread to the telomeres of other chromosomes [26]. Elongated telomeres of the survivors contained repeating patterns of marked and wild-type repeats, which varied among survivor strains, but within one survivor strain all telomeres had the same repeat pattern [27]. The authors proposed a roll-and-spread model in which a small extrachromosomal circle containing telomeric DNA is used in a rolling-

circle replication step (fig. 2C) to generate a long telomere by BIR, followed by invasion/replication of this telomeric pattern (fig. 2A) to the other telomeres in the cell.

Some mammalian cells that naturally lack telomerase activity or in which telomerase has been inactivated experimentally are able to grow and maintain their telomeres for many generations, indicating the existence of a telomerase-independent mechanism for telomere maintenance, which has been termed alternative lengthening of telomeres (ALT). As recently reviewed [1], many of the features described in ALT cells resemble those of the telomerase-negative type-II survivors of yeast, suggesting telomere maintenance by homologous recombination.

Chromosome breaks in *Drosophila*

Defined DSBs have been introduced in *Drosophila* somatic and germ-line cells by mobilization of P elements, which transpose by a cut-and-paste mechanism, leaving a DSB at the chromosomal donor site [28]. After DNA recession by exonuclease, the broken ends will sometimes invade homologous regions in the genome and copy them. This mechanism leads to targeted gene replacement around the original break with copies from elsewhere in the genome [29, 30]. The frequency of these break-induced gene-conversion events is below 1% but varies considerably with the cytological position of the target sequence, being higher near telomeres. In somatic cells, conversion events that lead to the loss of the *white* eye color gene can be detected as mosaic eyes [28]. These and similar experiments [31, 32] showed that in *Drosophila,* DSBs induce homologous recombination in somatic and germ-line cells and that *Drosophila* possesses a BIR pathway.

In a different approach, dispensable dicentric chromosomes were generated in the premeiotic cells of the male germ line [33]. This process led to chromosome breakage at mitotic anaphase, sending one broken end to each daughter cell. Although some of these broken chromosomes caused sterility and embryonic lethality, chromosomes with terminal deficiencies were readily recovered. When similar DSBs were generated from a dicentric chromosome in imaginal-disc cells of larvae, cell-cycle-checkpoint arrest and apoptosis ensued, the outcome depending on the proliferative activity of the cells [34]. Apparently, acquisition of a new telomere and resumption of proliferation does not occur nearly as frequently as in the germ line.

Heritable terminal deficiencies [35–40] have helped to characterize the telomere structure in *D. melanogaster*. Molecular and cytological analyses of some of these terminal deficiencies showed that the induced breaks were truly terminal and did not contain any new sequences at their breaks [37–39, 41, 42]. Surprisingly, these terminally deleted chromosomes can be maintained for many generations. The broken ends recede, however, at an apparently constant rate of about 75 bp per fly generation [36–39, 42, 43]. Because no cell-cycle-arrest response was elicited by these terminal deficiencies, they must possess a cap that protects them from being recognized by cell-cycle checkpoints, and capping must have occurred in the absence of specific DNA sequences at the chromosome ends.

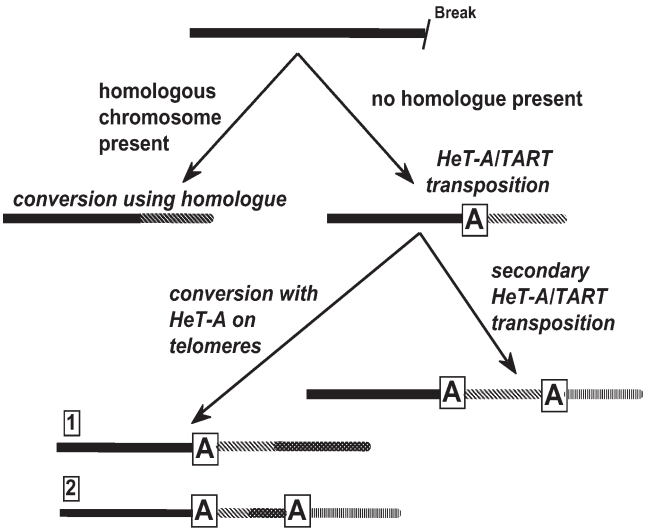

Figure 3. Observed elongation events at a terminal break in *Drosophila.* In the presence of a homologue, the broken end is extended by BIR. In the absence of a homologue, *HeT-A* or *TART* transpositions are most prevalent. Once a transposition has occurred, the added *HeT-A* element can become the target of secondary transposition events or can acquire other *HeT-A* element sequences by BIR using homologous *HeT-A* elements from other telomeres, giving rise to different types of arrangements depending on the location of strand invasion within the terminal retrotransposon array [1, 2]. The oligo(A) tails of the retrotransposons are indicated by boxed A's; hatched and stippled lines depict different copies of *HeT-A* or *TART* retrotransposons.

When the fate of X chromosomes with breaks in the *yellow* gene region was followed over several generations, two types of elongation events were observed (fig. 3). First, such ends in the male germ line experienced elongation by the addition of *HeT-A* transposable elements [43–45]. Transposition was calculated to occur at a rate that was just sufficient to balance the gradual loss of DNA from the chromosome ends, although genetic variants in the frequency of terminal additions occur that vary over a range of at least four orders of magnitude [40, 46, 47]. Taking advantage of the promoter activity in the 3′ UTR of *HeT-A* [48], Kahn et al. [40] observed *HeT-A* transpositions to a promoterless *yellow* gene at the break. Second, in the presence of the homologous X chromosome in females, terminal deficiency chromosomes were elongated by BIR [39]. In order to evaluate

the respective contributions of *HeT-A* transposition and gene conversion between *HeT-A* elements, Kahn et al. attached an ~300-bp *HeT-A* 3′ UTR to the broken chromosome end, which could then be the target for new transposition or engage in BIR with *HeT-A* elements at normal telomeres [40]. Of the 18 independent elongation events observed, 4–6 involved terminal conversion. The remaining elongation events were *HeT-A* transpositions. Transposition may therefore be the dominant mechanism for extension of broken chromosome ends in *Drosophila,* but in the presence of sequences with homology elsewhere in the genome, BIR also contributes significantly.

Species that lack telomerase-generated repeats

Drosophila melanogaster is the best-documented example of an organism without telomerase; chromosome breaks are never healed by the addition of telomerase-generated repeats, and a variety of oligonucleotide probes representing telomeric repeats do not hybridize to genomic DNA or to chromosome ends [49–51]. Instead, *D. melanogaster* telomeres do hybridize to DNA fragments from the non-long-terminal-repeat (non-LTR) retrotransposons, *HeT-A* and *TART* [41, 42, 52, 53]. Moreover, telomerase assays failed to detect any activity [54], and the genome lacks a gene resembling telomerase [55].

Clearly, several independent approaches are required to determine with reasonable confidence the absence of a telomerase-based elongation mechanism in any species. Negative evidence is difficult to interpret, and the absence of a hybridization signal with a suspected telomere repeat alone is not sufficient to prove the absence of telomerase-generated repeats in a particular species. This problem is exemplified in the case of some *Aloe* and *Allium* species. Their telomeres failed to hybridize to the typical plant-type telomere probe $(TTTAGGG)_n$ [56–58], but at least in *Aloe,* the telomeres were shown later to hybridize with the vertebrate-type $(TTAGGG)_n$ repeats [59]. Moreover, a variety of different oligonucleotide templates must be tested to demonstrate the absence of telomerase activity in an organism, as was done in several insect species [54]. A negative result does not exclude the possibility that an unusual telomeric repeat is synthesized by a telomerase in the species under investigation. Finally, cloning or polymerase chain reaction (PCR) amplification of chromosome termini can corroborate results obtained by the other methods and provide positive identification of terminal sequences, but clones from chromosome ends must be distinguished from those originating from DSBs. Although none of these techniques is perfect, combined results from various approaches, if consistent, can provide a good answer as to whether a particular species might use telomerase.

With a $(TTAGG)_n$ probe, hybridization signals were found at chromosome ends in the silkworm [50] and other lepidopteran species [60, 61] and in several ant species [62]. In a more extensive survey the TTAGG probe labeled telomeres in a variety of species from six insect orders [49], but the TTAGG motif was found in only 5 of 12 beetle species analyzed [63]. It is not known whether telomere elongation in these species is indeed independent of telomerase. The detection of telomerase activity in tissue extracts of crickets, cockroaches, potato hornworm and swallowtail butterfly strongly suggests that the pentanucleotide repeats that were observed by fluorescence in situ hybridization (FISH) are telomerase generated [54].

None of the tested dipteran species cross-hybridized with the $(TTAGG)_n$ probe that reacted with most other insect species [49, 50]. In fact, all dipteran species whose telomere DNA structure has been investigated so far appear to carry telomeric DNA sequences that do not resemble telomerase-generated repeats. The telomeres of the midge *Chironomus* have been studied extensively. Its chromosome ends consist of 50–200-kb blocks of complex satellite repeats, which are present at seven of eight chromosome tips [64–68]; the tip of the telocentric fourth chromosome harbors instead the kinetochore and other types of repetitive sequences [68, 69]. Different telomeres contain different subfamilies of repeats, but these subfamilies also show considerable variation in distribution at the same telomere among different individuals in the same stock [65, 70]. Subfamily D3, which may be able to form stable hairpins, is consistently located most distally, as indicated by Bal31 digestion [71], terminal tailing and PCR, and may extend all the way to the ends of the chromosomes [72]. *Chironomus* chromosome ends also lack the long 3′ single-strand overhangs found typically at telomerase-maintained telomeres [73]. Without a reporter sequence embedded in the telomeric repeat array, it is difficult to demonstrate telomeric recombination unequivocally. Recently, extrachromosomal copies of telomeric repeats have been reported in *Chironomus* [74], which are present in >20-kb DNA-RNA complexes. The origin of these complexes and their relationship, if any, to telomere recombination are unknown. They may originate from transcripts of telomeric repeats, which are converted into DNA by a reverse transcriptase that has been found associated with some telomeres [75]. Alternatively, extrachromosomal telomeric DNA repeats might be products of rapid telomere shortening or may be used in telomere extension.

Telomere structure has also been studied at the 2L telomere in the mosquito *Anopheles gambiae* with the aid of an inserted transgene. This telomere contained long stretches of a complex 0.8-kb repeat satellite [76, 77], but only the transgene was observed at the end, suggesting that mosquitoes do not use retrotransposons or

telomerase to extend their broken chromosome ends. The distal end of the transgene receded at a rate of 55 bp per generation, suggesting that the transgene was indeed terminal [78]. In 2 years (~100 generations), some 2L chromosome ends had become elongated by duplicating part of the integrated pUChsneo plasmid, suggesting recombination between homologous 2L telomeres [79]. This mechanism may also be used in wild-type telomere elongation in *A. gambiae*, although proof is still lacking.

The absence of simple telomeric repeats and the presence of long arrays of complex tandem repeats at the telomeres of dipteran insects have led to the hypothesis that telomerase has been lost in an ancestor of dipteran insects [80, 81]. Elongation mechanisms such as BIR may be viable means of telomere elongation in these insects. Apparently, only *D. melanogaster* and its closest relatives [82, 83] have recruited retrotransposable elements for telomere elongation. Even in *Drosophila virilis*, a fairly distant relative of *D. melanogaster*, a satellite array with a complex nucleotide composition and a repeat unit of about 370 bp was found at the ends of all chromosomes as well as in many nontelomeric locations [84].

Telomere elongation in *D. melanogaster*

Chromosome length in *D. melanogaster* is probably maintained by transposition of telomere-specific non-LTR retrotransposons, *HeT-A* and *TART*, with attachment of the elements to the chromosome end by their 3′ oligo(A) tails [85]. The structure of these elements has recently been reviewed in detail [86, 87]. Natural chromosome ends in this species consist of tandem arrays of *HeT-A* and *TART* elements with their oligo(A) tails toward the centromere. Immediately proximal to the terminal retrotransposon array lie several kilobases of a complex subterminal satellite, termed telomere-associated sequence (TAS) [41, 42, 88]. TASs from different chromosomes exhibit sequence similarities and some limited cross-hybridization [88]. *HeT-A* elements probably use the promoter located in their 3′ UTR [40, 48] to transcribe the downstream element in the telomeric array, and occasional long readthrough products may give rise to transpositions of multiple elements [89]. Full-length transcripts have been detected by Northern blots, but in contrast to *HeT-A,* which is transcribed asymmetrically, *TART* appears to be transcribed from both DNA strands [90]. This unusual transcription pattern generates transcripts that resemble those of LTR retrotransposons with terminal redundancy, as its 5′ sequences are identical to its 3′ sequences. Thus, because of its unusual head-to-tail arrangement at telomeres, *HeT-A* has characteristics of an intermediate stage between non-LTR and LTR retrotransposons [48, 81, 91].

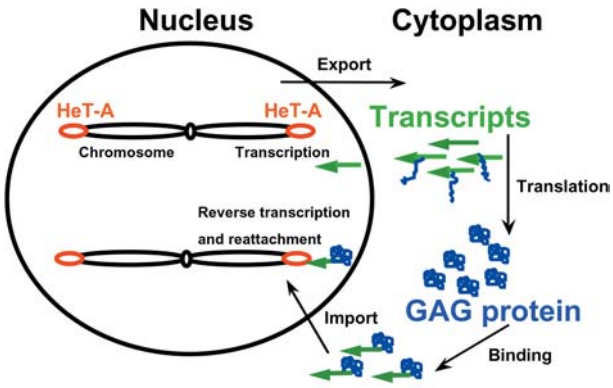

Figure 4. Model of nuclear and cytoplasmic events in *HeT-A* (or *TART)* transposition. Telomeric retrotransposon elements are transcribed from the promoter of the more distally located element, and transcripts leave the nucleus to be translated. The GAG-like protein binds to the transcript, and this RNP complex reenters the nucleus, attaches to a chromosome end and becomes a substrate for a reverse transcriptase, leading to the addition of a new copy of *HeT-A* or *TART.* Reprinted with permission from Landes Bioscience and Kluwer Academic/ Plenum Publishers, 2002, see [87].

Little is known about the actual transposition mechanism of *HeT-A* and *TART* or its regulation. Figure 4 shows steps in the proposed transposition cycle of a *HeT-A* (or *TART*) element. The transcripts leave the nucleus to serve as messenger RNA (mRNA) for the translation of the element-encoded polypeptide(s). The *HeT-A*-encoded GAG-like protein plays an important role in the transposition of the retroelement and, like viral nucleocapsid GAG proteins, may facilitate entry of viral nucleic acid into the nucleus [92], where it forms distinct foci [93]. After entry into the nucleus, recognition of a telomere by the RNA intermediate might be mediated by a protein-protein interaction between the GAG-like protein and the terminal end-binding complex.

As a first step toward understanding the regulation of the *HeT-A* promoter in situ, we used a repressed *white*$^{+}$ eye color transgene, *P{w*^{var}*}*, inserted between *HeT-A* and TAS at the *2L* telomere [46]. Phenotypic eye color variants arose spontaneously in the germ line with high frequency, and molecular analysis of these variants revealed that transgene expression was correlated with alterations in the telomere region itself. Higher expression of the transgene was associated with *HeT-A* and *TART* additions, and lower expression with loss of *HeT-A* and *w* sequences from the chromosome end. This result, in conjunction with an earlier observation that TAS suppresses *w* in nontelomeric transgenes [94], implicates TAS in telomeric silencing and suggests that TAS silencing spreads distally, toward the *HeT-A* array, and may serve to regulate *HeT-A* transcription and transposition and, thus, telomere elongation.

Expression of the telomeric *white* reporter gene downstream of a long *HeT-A* array also increased in response

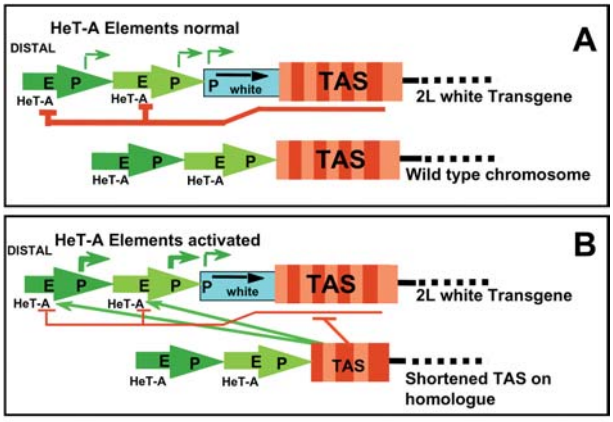

Figure 5. Features of our telomere-position-effect model include *cis*-acting silencing (red) of the *white* transgene by TAS and *cis*-acting activation by *HeT-A* (green). The *w* transgene expression at the telomere is influenced negatively by TAS and positively by the promoter/enhancer activity in the terminal *HeT-A* array (*A*). When TAS on the homologous chromosome is shortened (*B*), expression of the *w* transgene at the telomere is increased, either by positively influencing promoter activity in the terminal *HeT-A* array (green) or by negatively influencing TAS silencing on the homologous chromosome (red). E, putative enhancer region on *HeT-A* element; P, promoter region on *HeT-A* and *white*.

to deletion of some or all of the TAS on the homologous chromosome [95]. Thus, expression of a subtelomeric reporter gene is influenced by the telomere structure in *cis* and *trans*. Deficiencies of TAS on the homologous telomere may disrupt a pairing-dependent repression mediated by TAS, induce promoter activity of a *HeT-A* 3′ UTR, or inhibit the *cis*-silencing of this promoter (fig. 5). These observations led to the proposal that telomeric position effect observed in *D. melanogaster* reflects a telomere-length-control mechanism [96, 97]. Variegated expression of reporter genes at telomeres may, thus, reflect a competition between the repressive effects of TAS and the stimulating effects of *HeT-A* promoters. In other words, the mechanisms involved in telomere length regulation in *Drosophila* may comprise the underlying forces that manifest themselves as telomeric silencing. Thus, TAS may play an important role in regulating telomere elongation by controlling *HeT-A* promoter activity.

Chromosome capping in *Drosophila*

Stereotypical tandem repeats at chromosome ends, either generated by telomerase or maintained by homologous recombination, can maintain a specialized nucleoprotein configuration even while the arrays themselves are expanding or contracting. Unless the array reaches a critically short length, a functional telomere cap can be formed between repeating nucleotide sequences and specific proteins that bind to them. At *D. melanogaster* telomeres, however, *HeT-A/TART* arrays are much more complex and variable. Further, terminal chromosome deficiencies exhibiting completely unrelated terminal nucleotide sequences are stably transmitted in the germ line and appear to be capped. These observations suggest the existence of sequence-independent capping mechanism in *Drosophila* [36, 37].

Because telomere-telomere associations are indicative of a defective telomere cap, Gatti et al. [98] screened for this phenotype and first identified the *UbcD1* gene, encoding a ubiquitin-conjugating enzyme. The ubiquitination target protein is still unknown. It may not be directly involved in chromosome capping but be required for maintaining proper chromosomal orientation during interphase, by mediating telomere associations, and need to be degraded during mitosis.

The first identified potential component of the *Drosophila* capping complex is the heterochromatin protein, HP1. Although the bulk of HP1 is localized on the mostly heterochromatic fourth chromosome and in the pericentric heterochromatin, it also binds to natural chromosome ends and terminal deficiencies independent of the presence of *HeT-A* and TAS sequences [99]. HP1 is maternally deposited in the embryo, one of the postulated features of a capping protein, where it forms complexes with the origin-recognition complex (ORC) as well as with various other proteins [100], one of which has recently been identified as HP1/ORC2-associated protein, HOAP [101]. Defects in telomeric capping often manifest themselves as an increased frequency of telomere-telomere fusions detectable as anaphase bridges. Because this phenotype is found in HP1 mutants [99] as well as in the HOAP mutant *caravaggio* [M. Gatti, personal communication], these two proteins are implicated in telomere capping. Moreover, HP1 mutations increase by more than 100-fold the transposition frequency of *HeT-A* and *TART* elements to broken chromosome ends but do not affect terminal gene conversion [102]. It is unclear whether the increased transposition rate is due to a failure in capping efficacy due to partial loss of HP1, making the chromosome end more accessible, or to a derepression of *HeT-A* promoters, leading to an observed increase in *HeT-A* transcript levels [102]. Although HP1 mutations are suppressors of centromeric position effect variegation [103], they do not affect telomeric silencing [104]. This observation indicates that HP1 may not be a component of a hypothetical TAS-binding complex, which may be responsible for silencing at telomeres [94, 96, 97]. As HP1 has no direct DNA binding ability itself, an unidentified telomere-binding protein probably recruits HP1 to the chromosome end. One candidate for this recruitment may be HOAP, as in the *caravaggio* mutant that lacks full-length HOAP, HP1 levels are reduced at diploid chromosome ends [105], and HP1 appears absent from polytene chromosome tips [M. Gatti, personal communication]. Another potential component of the telomeric cap may be encoded

by the *tef* gene. Mutations in this gene also give rise to telomere fusions that are not resolved in anaphase, causing genome rearrangements and apoptosis [106].

Conclusion

Telomere elongation by mechanisms other than telomerase are widespread among eukaryotes and can be observed in a variety of organisms when the capping function of the telomere is compromised. Telomerase is very efficient in maintaining existing telomeric repeats but much less efficient in de novo attachment of such repeats. Therefore, chromosome ends lacking these repeats are more likely to undergo recombination events or become targets of transposition. In organisms that have lost telomerase activity either through experimental manipulation or during the course of evolution, these pathways are efficient enough to ensure survival.

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