Differential Maintenance of DNA Sequences in Telomeric and Centromeric Heterochromatin

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ABSTRACT

Repeated DNA in heterochromatin presents enormous difficulties for whole genome sequencing; hence, sequence organization in a significant portion of the genomes of multicellular organisms is relatively unknown. Two sequenced BACs now allow us to compare telomeric retrotransposon arrays from *D. melanogaster* telomeres with an array of telomeric retrotransposons that transposed into the centromeric region of the Y chromosome >13 Myr ago, a unique opportunity to compare the structural evolution of this retrotransposon in two contexts. We find that these retrotransposon arrays, both heterochromatic, are maintained quite differently, resulting in sequence organizations that apparently reflect different roles in the two chromosomal environments. The telomere array has grown only by transposition of new elements to the chromosome end; instead, the centromeric array has grown by repeated amplifications of segments of the original telomere array. Many elements in the telomere have been variably 5'-truncated apparently by gradual erosion and irregular deletions of the chromosome end; however a significant fraction (four and possibly five or six of 15 elements examined) remain complete and capable of further retrotransposition. In contrast, each element in the centromere region has lost 40% or more of its sequence by internal, rather than terminal, deletions and no element retains a significant part of the original coding region. Thus the centromeric array has been restructured to resemble the highly repetitive satellite sequences typical of centromeres in multicellular organisms, whereas, over a similar or longer time period, the telomere array has maintained its ability to provide retrotransposons competent to extend telomere ends.

INTRODUCTION

The wealth of genome sequences now available has revealed much about genome organization and how this organization has evolved. These sequences have greatly extended our understanding of the ways in which transposable elements have added to and shaped eukaryotic genomes (BOURQUE 2009; CORDAUX and BATZER 2009; SLOTKIN and MARTIENSSEN 2007). For most eukaryotes, however, a significant portion of the genome is still poorly understood. This portion is the heterochromatin, which makes up about a fifth of the human genome and a third of the Drosophila genome (HOSKINS *et al*. 2007). Heterochromatin is very rich in transposable element sequences and it would be especially interesting to understand how these sequences are related to the properties that distinguish heterochromatin from euchromatin. However the large number of highly repeated and rapidly evolving DNA sequences in heterochromatin presents problems for accurately assembling sequences long enough to give a complete picture of the organization of these elements and their possible roles in specific heterochromatic regions such as centromeres and telomeres.

We are studying the three non-LTR retrotransposons that maintain the length of Drosophila telomeres, *HeT-A*, *TART,* and *TAHRE*. These elements transpose by means of a poly(A)+ RNA which is reverse transcribed directly onto the end of the chromosome (PARDUE *et al*. 2005). Successive transpositions form long arrays of head-to-tail repeats. These repeats are analogous to the repeats that telomerase adds in most other organisms except that the Drosophila repeats are copies of retrotransposons, three orders of magnitude longer than the repeats added by telomerase. Recently we analyzed a BAC and a finished scaffold of equal quality

containing sequence from two *D. melanogaster* telomeres. These sequences gave us our first overview of both the organization of transposable elements within a telomere (GEORGE *et al*. 2006) and, now, the mechanisms by which these telomeres are maintained.

Although the telomeric retrotransposons appear capable of transposing only onto chromosome ends, either natural telomeres or broken chromosomes, *HeT-A* DNA was found to hybridize to the centromere region of the *D. melanogaster* Y chromosome (AGUDO *et al*. 1999) and later shown to colocalize with antibody to centromere-specific histone on the Y chromosomes of other members of the melanogaster species subgroup (BERLOCO *et al*. 2005). Thus, *HeT-A*-related sequences appear to have been maintained at the centromere of the Y for over 13 Myr, even though the structure of the chromosome has diverged so that the Y is now metacentric in some species and telocentric in others. MENDEZ-LAGO *et al.* (2009) have recently reported sequence of a BAC from this *D. melanogaster* Y centromere cluster.

The assembled sequences of the telomeric BAC and the centromeric BAC give the first opportunity to examine arrays long enough to allow us to analyze the organization, maintenance, and evolution of these retrotransposon arrays in two different heterochromatic environments. Comparison of the telomeric and centromeric sequences reveals that *HeT-A* arrays in telomeric heterochromatin are maintained very differently from those in centromeric heterochromatin; each is now structured in ways that appear to be compatible with their different roles at the telomere and the centromere. It is frequently said that transposable elements have done much to shape eukaryotic genomes, in these cases, the genome is shaping the elements.

RESULTS AND DISCUSSION

HeT-A elements in telomeric heterochromatin

Sequences analyzed. Our comparison is based on a telomeric BAC from 4R plus sequence from directed finishing of a scaffold from the telomere of chromosome XL, reported previously, but with some revisions in annotation. Both the 4R and the XL sequences begin in their assembled chromosome and extend into the telomere, thus showing the precise relationship between these telomere arrays and the rest of the genome (GEORGE *et al*. 2006). Neither sequence extends to the distal end of the telomere but together they give nearly 100 kb of telomeric *HeT-A*/*TART* array (76 kb from 4R and 20 kb from XL). Importantly, both include the most proximal elements of the array. Because telomere elements are added sequentially, the most proximal elements must be the oldest; thus, these arrays present the most accurate history available of *D. melanogaster* telomere maintenance.

Neither the 4R nor XL sequences have non-telomeric elements in the telomeric array except for a small transition zone at the proximal edge of the array where there are some fragments of non-telomeric elements. We do not include these transition zones in our discussion of telomere arrays. Figure 1 gives an overview of the organization of *HeT-A* sequences in the telomere array and transition zone of 4R. The sequence of this segment of the BAC is compared to the sequence of the canonical *HeT-A,* 23Zn-1, which is diagramed on the X axis of the dotplot. Although none of the elements in the array belong to the same subfamily as the canonical element, all show

linear similarity except for small gaps or repetitions in the 3'UTR. Each complete or partial *HeT-A* in the array is intact at the 3' end. Essentially all sequence loss has been at the 5' end. Complete elements match the entire 5' end of the canonical *HeT-A*. Two elements that have mildly truncated 5'UTRs are grouped with partial elements because we do not know whether they contain 5'UTR sequence required for transposition competence.

HeT-A elements are much more abundant than *TART* elements in all *D. melanogaster* genomes we have analyzed. Consistent with this, there are very few *TART* elements in the 4R array and none in the shorter XL sequence. Because we are making comparisons with a segment of the centromere array that consists entirely of *HeT-A*, we have not included *TART* data in the following analyses and discussion. Empty space in Figure 1 contains complete or partial *TART*s. The third (very rare) telomeric retrotransposon, *TAHRE*, is also omitted from discussion because it is not found in any of the arrays considered here.

Each telomere array is a chronological record of events at the end of the chromosome. The studies reported here analyze the 4R and XL data to investigate sequence management within an intact Drosophila telomere. These sequences present a unique opportunity: the BAC and the sparser, but still useful, finished scaffold data are the first long sequences that give the exact order of elements in the telomere and their relationship to the rest of the genome;. This relationship is informative because *HeT-A*, *TART*, and *TAHRE* transpose only onto the ends of chromosomes so that the order of elements in an array reflects the order of transposition onto the chromosome, with the oldest elements at the proximal end. Rearrangements and imprecise recombination are

ruled out by the large scale integrity of all the elements present; the full length elements evidence replicative capability and even the partial elements are simply 5'-truncated with no evidence of sequence rearrangement or decay.

The sequence at the 5' end of each internal element is a record of the sequence that remained on a terminal retrotransposon when that element was capped by a newly transposing element, and thus demoted to an internal position in the telomere. Comparing that capped sequence with the 5' end of its presumed RNA template gives an estimate of the amount of sequence lost while that element served as the end of the chromosome. (This is a maximum estimate because some sequence could have been lost in transposition.)

The only measurements of the rate of end erosion and the addition of new elements on Drosophila chromosomes have come from broken chromosomes that have lost all telomeric and subtelomeric DNA. Such chromosomes shortened gradually by about 70 nucleotides per fly generation (BIESSMANN *et al*. 1990; Levis 1989; Mikhailovsky et al. 1999), calculated to represent an average loss of 2-3 nt per cell generation (Biessmann et al. 1990). The rate at which a broken end is healed by addition of *HeT-A* varied from $< 2x10^{-5}$ to $2x10^{-3}$, depending on the background genotype (KAHN *et al*. 2000). Thus, broken chromosome ends show a fairly regular slow loss of end sequence accompanied by infrequent addition of large retrotransposons; *HeT-A* is ~6 kb, *TART* and *TAHRE* are 10-12 kb.

The 4R and XL sequences give us the first opportunity to analyze the turnover of sequence on established telomeres. For this analysis we first discuss two indicators of sequence loss in the telomere arrays. 1) the Tags (see following section) of non-

essential sequence on the 5’ end of each *HeT-A* RNA, and 2) the distribution of complete and 5’ truncated elements in the telomere array.

These data fall into two classes. Qualitative observations describe the nature of the processes governing telomere maintenance and renewal; these observations strongly constrain models based on numerical analysis. In turn quantitative statistical analyses help in distinguishing and judging competing conclusions. As discussed below, we conclude that maintenance of established telomeres involves at least three processes acting in concert to maintain relatively stable conditions: relatively short range terminal erosion, long range terminal deletion, and irregular transpositions.

5’ Tags reveal slow sequence erosion of the telomere end. Tags are short sequences added to the 5’ end of *HeT-A* RNA by *HeT-A*‘s unusual promoter which initiates transcription within the 3’UTR of the upstream element (DANILEVSKAYA *et al*. 1997; TRAVERSE *et al*. 2010). The resulting Tag of upstream sequence becomes a de facto extension of the 5’UTR and is reverse transcribed with the rest of the RNA when the element transposes, providing expendable sequence to buffer loss of essential 5’ sequence from chromosome end erosion. Part or all of a Tag can be eroded while it forms the end of the telomere (Fig. 2A). When a new element transposes to cap the chromosome end, erosion of the capped Tag is halted, leaving a partial Tag. Repeated transcription of an element will add a new Tag to any already on its 5’ end. Thus, an element can have a 5’ string of variably truncated Tags - evidence that it has transposed multiple times (Fig. 2B). Tags are the hallmark of complete *HeT-A*s, which carry a string of them. We note that there is evidence to suggest that erosion can at times continue into the 5’UTR (see the discussion of truncated *HeT-A*s below).

Tags are the best indicators of nucleotide loss on the end of an established telomere. There is only now a statistically robust sample of Tags and, because Tags are born in discrete short lengths, their end erosion is directly measureable. (It is frequently convenient to differentiate between a Tag's 3'-oligoA sequence, its "Tag-tail", and its more 5' sequence, the "bare Tag"). The initial length of each bare Tag is determined by its transcription start site, which can be 93, 62, or 31 nt from the 3'end of the element serving as a promoter; the 3'-oligoA of the promoting element then forms the tail of this new Tag (TRAVERSE *et al*. 2010). Thus the longest Tag should have a 93 nt "bare Tag" plus its tail, the Tags in our arrays are all much shorter than this (Fig. 2A).

If end erosion is regular and averages ~ 70 nt per fly generation, as found for broken ends, most Tags should last <1 generation, but complete elements typically have several Tags in various states of erosion (Fig. 2B). Strings of partially eroded Tags indicate that these expendable sequences provide enough protection to allow a significant number of elements to survive with intact 5' ends.

Tag Properties. On average**,** our Tags are surprisingly short (Fig 3A). Their median length, including the oligoA tail, is 11 nt, mean 14.0 with the 95% Confidence Interval of the mean 10.7 - 17.3 nt. Furthermore, the very shortest Tags are over-represented (18% have the sequence TAAA but contain <5% of the total Tag sequence), suggesting that the rate of sequence loss is reduced as Tags are eroded toward their oligoA tail. There is also one very long Tag (68 nt) which is a distinct outlier; the next longest is 38 nt. The paucity of long Tags may be evidence that the two distant transcription starts, -93 and -62, are very rarely used. Alternatively, the longest Tags may be subject to

more severe erosion; in fact, there may be an accumulation at the transition to bare lengths about 31 nt. The evidence for this conjecture is too weak to assert with confidence.

We note that the narrow limit on Tags per string (5-9) and Tag string length (69-161 nt) indicates that erosion is regulated to balance transposition because we find neither intact HeT-As without Tags, nor strings that have grown without limit, as they would if not effectively pruned.

HeT-A oligoA tails (mean 8.3 nt, 95% Confidence Interval 4.6-12.1 nt) are very short compared to *TART* oligoA tails (18.3 nt, range 13-23 nt). Because *HeT-A* oligoA tails give rise to Tags, and because *TART* does not utilize Tags to protect its 5'end, this suggests that *HeT-A* oligoA length is one adaptation to help control the overall length of *HeT-A* Tag strings. With *TART*-like tails, the average Tag and Tag string would more than twice as long,

These analyses show that erosion of the telomere end is more complex than the relatively regular loss described by studies of broken chromosomes. It also shows that many, perhaps all, new transpositions occur before the terminal Tag has been completely eroded. In the maintenance of the extreme ends of chromosomes, there are at least two stochastic processes at work: relatively regular erosion of Tags and a mechanism that protects the very shortest ones, but it is important to recognize that Tag erosion is, nevertheless, a relatively closely regulated process.

The distribution of 5'-truncated elements in the telomere array provides evidence of sporadic terminal deletions. Within experimental uncertainties, the rate of terminal erosion measured from Tag lengths approximates that measured on broken

chromosomes. In contrast, sequence loss from 5'-truncated elements is on a much larger scale. There are eleven truncated *HeT-A*s in these arrays. All contain the 3'-most 150 nt (which is almost completely conserved among *HeT-A* subfamilies) but they differ significantly in the amount of sequence lost from the 5' end (Fig. 3). Their lengths are broadly distributed between 5892 bp and 241 bp, showing no correlation with position in the array. The longest two of these *HeT-A*s have partial 5'UTRs, the next longest three contain partial ORFs; all others have only 3'UTR sequence. All have enough 3'UTR to provide promoter activity for a downstream neighbor, although the 274 bp element would provide only weak activity.

If the sequence loss on a telomere occurs at the gradual rate measured for broken ends, and the eleven truncated elements here were unprotected by 5' capping Tags, then they would have resided on the end of the telomere for times ranging from one fly generation (the longest truncated element) to 81 fly generations (the shortest element). Because the Tag analysis shows that elements frequently remain on the extreme end of the chromosome for less than one fly generation it seems unlikely that many of the more truncated elements result from gradual end erosion. Instead we favor the idea that truncation can result from terminal deletion. These terminal deletions may occur at many places within the array, leading to occasional rebuilding of all or part of the array.

Sequence loss from the longest truncated element (asterisk in Fig. 1) falls within the range of sequence loss measured by Tag erosion, suggesting that it was produced by the same erosional process, rather than terminal deletion. With much lower probability the same might be true of the second longest (asterisk in Fig. 1). It is also possible that one or both of these elements are transposition-competent since each retains part of its

5’UTR. (Unlike the typical non-LTR element, *HeT-A* does not have its promoter in the 5’UTR, thus these two elements have not lost essential promoter sequence.) However, 5’UTR sequence might also have other functions, such as directing second strand synthesis during transposition. Until more is known about activities of the 5’UTR we cannot know whether either of these elements is competent to produce new *HeT-A* transpositions. Therefore we do not include them as complete elements.

Although Drosophila telomeres have telomere-specific retrotransposons rather than telomerase, their telomeres appear to be functionally analogous to telomeres in other organisms. The use of occasional terminal deletions to maintain telomere length is another point of similarity with other organisms. The first evidence that terminal deletion is used to regulate telomere length came from studies of (TRD) terminal rapid deletion in budding yeast (BUCHOLC *et al*. 2001; LI and LUSTIG 1996; LUSTIG 2003). More recently mammalian telomeres have been shown to utilize a similar mechanism (PICKETT *et al*. 2009; WANG *et al*. 2004. These examples show that loss of long segments of telomeres can be part of the regulation of telomere length. We suggest that similar rapid losses are also utilized by Drosophila telomeres, although the mechanism of the deletion may be different. Terminal deletions in mammals and years are most likely due to homologous recombination between their short, identical telomere repeats. It is less likely that such recombination is a major cause of deletion in the more complex repeats of Drosophila telomeres.

It is possible that some *HeT-A* elements become truncated during the process of transposition (TRAVERSE *et al*. 2010). However, we suggest that most truncated elements in the arrays result from terminal deletion. Evidence of repeated loss of

complete telomere arrays, discussed below, suggests that large terminal deletions are not uncommon, especially if we consider that any terminal deletions not reaching into subtelomere regions would almost certainly have escaped detection.

Although Drosophila telomeres may not undergo terminal deletion by homologous recombination, there is evidence that they do undergo loss of complete telomere arrays and then rebuild by addition of telomere retrotransposons. The evidence is perhaps more convincing because some of it is a byproduct of investigations directed, not at chromosome ends, but at *P-element* expression. *P-elements* frequently insert in subtelomeric sequences and three inserts on the tips of X (MARIN *et al.* 2000), 3R (SHEEN and LEVIS 1994), and 2L (GOLUBOVSKY *et al.* 2001), were found to have terminal deletions removing the telomere array and part of the *P-element* inserted in telomere associated sequences. In most cases it appears that the P-element did not cause the deletion. Further evidence of long terminal deletions comes from studies of subtelomeric regions (KERN and BEGUN 2008; WALTER *et al.* 1995). These regions have high levels of gene presence/absence polymorphism not seen in the adjacent euchromatin. At least some of this structural polymorphism has been shown to be due to terminal deletions that have been healed by transposition of *HeT-A* (KERN and BEGUN 2008). Early studies of *lethal (2) giant larvae*, near the 2L tip (WALTER *et al.* 1995), and a recent, more extensive study of the tip of 3L (KERN and BEGUN 2008) have revealed deletions similar to those found in the *P-element* studies mentioned above.

Telomere arrays contain an unexpected number of complete elements with coding regions that show no signs of sequence decay. If terminal sequence loss

and new transpositions were relatively regular continuous processes, it might be expected that each element in the array would undergo approximately the same amount of 5'-truncation. As reported earlier (GEORGE *et al*. 2006), this is not what the arrays show. Complete *HeT-A* and *TART* elements are overrepresented in the 4R and XL telomere arrays. (This includes two complete *TART*s making up 24.6 kb of the 4R array. For simplicity they are not shown in Figure 1 and will not be considered here.) The 4R telomere sequence has three complete *HeT-A* elements and the most proximal element in the XL array is also complete. None of these elements shows evidence of sequence decay. Each has a complete 5'UTR with an associated cluster of Tags, indicating that it has transposed several times.

HeT-A elements have a single ORF. It encodes a Gag protein involved in localization to telomere regions and apparently required for transposition (PARDUE *et al.* 2005; RASHKOVA *et al.* 2003; RASHKOVA *et al*. 2002). Complete coding regions in the *HeT-A* elements of the 4R and XL arrays identify four *HeT-A* subfamilies whose *gag* genes range from 2766 nt to 2856 nt (GEORGE *et al.* 2006). Most of the difference is in a length polymorphic region near the N-terminus of the protein. None of these polymorphisms interrupt the reading frame and all subfamilies share conserved sequences involved in specific interactions with other telomeric Gags (FULLER *et al*. 2010).

Even the three truncated *HeT-A gag* genes in these arrays show no degradation except for loss of 5' sequence. And although some *gag* sequences lie in the most proximal, and therefore the oldest, part of the 4R and XL telomeres, they show no sign of sequence decay, even though they should no longer be under selection for function.

This suggests that these arrays turn over more frequently than other chromosomal regions.

This study suggests that at least three processes may be operating in telomere arrays: slow erosion, terminal deletion and irregular transposition. Like Tag erosion**,** transposition cannot be purely stochastic. It must be regulated in concert with end erosion and relatively frequent terminal deletion to control telomere length in response to environmental cues, to preserve a reservoir of replicatively competent HeT-A elements, and to balance the fact each transposition adds orders of magnitude more sequence than the loss measured from Tag erosion (*HeT-A,* 6 kb, or *TART* or *TAHRE*, 10-12 kb).

It appears that telomere retrotransposons have two major functions. They provide telomere-specific DNA, analogous to the telomere-specific repeats produced by telomerase. They also maintain a population of functional elements capable of adding to the transposon array. The first function can be fulfilled by truncated elements but the second function requires that some elements escape 5' truncation and sequence decay. Our studies show that the 5' Tags could provide protection against gradual terminal erosion, at least for elements that do not remain in the terminal position very long. Terminal deletions could maintain a more regular telomere length in spite of the very long additions added by each transposition. Perhaps more importantly, they would remove decayed elements, allowing replacement by transposition-competent elements when the deleted telomere is regenerated by new transpositions.

We propose that together the processes of slow erosion, terminal deletion, and irregular transposition maintain an environment that forms telomeric heterochromatin

and also assures a supply of new *HeT-A* elements competent for telomere-specific transposition to maintain chromosome length

HeT-A ELEMENTS IN CENTROMERIC HETEROCHROMATIN

Sequences analyzed. The BAC sequenced by MENDEZ-LAGO *et al.* (2009), contained part of a telomere array that apparently transposed into the centromere region of the Y chromosome more than13 Myr ago (BERLOCO *et al*. 2005). This conserved localization suggests that the *HeT-A* cluster has some role at the centromere, possibly forming the kinetochore, affecting sister chromatid cohesion, or maintaining the heterochromatic environment.

MENDEZ-LAGO *et al*. (2009) suggest that the sequence initially consisted of nine telomere retrotransposons in a typical telomeric head-to-tail array. Five retrotransposons, four *HeT-A*s and one *TART*, at the distal end on the telomere were all extremely 5'-truncated. The remainder of the founder array consisted of four complete *HeT-A* elements, numbers six, seven, eight, and nine in their notation (see Table 1). This founder sequence could have been either a Y chromosome telomere that moved to the interior by an inversion, or a segment of telomere from another chromosome inserted into the Y, which has a record of accepting sequence from other chromosomes (KOERICH *et al*. 2008).

The proposed nine element founder sequence that moved into the Y would have been about 30 kb long. In the Y chromosome it has grown into a large sequence cluster: the BAC contains 159 kb of Y chromosome sequence and the *HeT-A* cluster is truncated by cloning on both ends of the BAC. Part of the growth of the cluster is due to

the insertion of members from seven families of non-telomeric transposable elements. Nevertheless, the majority of the expansion has come from amplification of regions within the original array. MENDEZ-LAGO *et al*. (2009) propose that this amplification came about by a series of events involving different sections of the founder sequence.

The amplifications divided the telomere sequence into two different kinds of arrays (Fig. 4A). The severely truncated elements formed a 3.1 kb repeat that has been amplified to make up more than 100 kb of relatively homogeneous simple sequence repeats of the type classified as satellite DNA. This section is named the 18HT satellite.

The four complete *HeT-A* elements underwent a series of head-to-tail amplifications of different regions within their array to give ten elements and an eleventh truncated by the cloning procedure. These elements, with the transposable elements inserted in them, now make up a complex set of repeats that stretches over more than 60 kb of the BAC. We refer to this region as the *HeT-A* array and designate the current set of elements as A through K (see Table 1 for their ancestral derivation as proposed by MENDEZ-LAGO *et al*. 2009).

During their time on the Y chromosome, these *HeT-A* sequences have been conserved very differently from the *HeT-A*s in telomere arrays. An overview of sequence from the centromeric BAC (Fig. 4) shows that *HeT-A* sequences are much more fragmented than they are in the telomeric BAC (compare Fig. 4A with Fig. 1A).

Amplification events are not seen in telomere arrays. In telomere arrays, each element is uniquely defined by combinations of subfamily sequence, 5’ truncation, presence or absence of Tag sequences, and length of the 3’ oligoA tail. These characters allowed MENDEZ-LAGO *et al*. (2009) to identify elements amplified in the

centromeric sequence. Similar amplifications have not been detected in telomere regions. Thus the amplification events provide the first evidence that the *HeT-A* array is being maintained differently in its centromeric position.

Full-length *HeT-A* elements in the centromeric array have undergone extensive internal deletions. The ten centromeric elements derived from ancestral intact elements six through nine have undergone extensive sequence changes. Our analyses of this sequence (Fig. 4B) shows that each of these centromeric elements has lost some 40% or more of its sequence, and none would encode the Gag protein thought to be necessary for *HeT-A* transposition. In the original report these elements are described as decayed, however our comparisons with *HeT-A* suggests that the changes are perhaps more accurately described as restructuring rather than decay because they are not entirely random.

The four dotplots (Fig. 4B) comparing individual centromeric elements to canonical *HeT-A* give a representative view of the changes in this array. In contrast to the telomere elements where loss is always from the 5' end, each centromere element has several large internal deletions scattered through its sequence. There has been little rearrangement of the remaining sequence, most of which is collinear with the canonical *HeT-A* and has relatively few inversions and rearrangements. Surprisingly, the only regions that are conserved in every centromere element are the extreme 5' and 3' ends.

Comparisons of these elements show that many deletions are shared with siblings derived from the same amplification. Thus these ten elements and the partial element have become a complex array of repeats. Because amplification events apparently involved more than a single unit, higher orders of repeat arise, as can be seen in the full

length view shown in Figure 4A. A more specific example of a higher order repeat, elements H, I, J, and K, is shown in Figure 4B. H and J are nearly identical while the alternating elements I and K are also nearly identical but quite different from their flanking elements (H and J).

As a result of both sequence loss and nucleotide changes, these *HeT-A* elements have lost much of their protein coding capacity. The longest open reading frames in these elements range from 246 to 558 nucleotides while the shortest complete *HeT-A* *gag* gene is 2766 nt.

The centromeric *HeT-A* array contains several non-telomeric transposable elements.

At the telomere, non-telomeric elements are found only in small transition zones at the junction between the telomere array and the rest of the chromosome; elements in the transition zones are only fragments. In contrast, the centromeric BAC contains non-telomeric elements from seven different families, three in the 18HT sequences and four in the *HeT-A* array. In the *HeT-A* array, one element, the non-LTR retrotransposon *F*, arrived early in the expansion of the sequence and was included in two of the amplification events. More recent arrivals, *mdg1*, *diver*, and *1731*, are present as single copies.

The LTR retrotransposon *1731* is especially interesting. It is completely intact and its two LTR sequences are identical. This element is a member of the *1731* subfamily in which the *gag* and *pol* reading frames are fused to produce an ORF of 3852 nt (KALMYKOVA *et al*. 1999). This entire reading frame is open and has 100% nucleotide identity with other elements in the database. Therefore, this element is potentially

active. Other studies have suggested that there is at least one active *1731* on the Y chromosome. The *1731* fused Gag-Pol protein is expressed in testis (KALMYKOVA *et al*. 2004), where Y chromosomes are genetically active. Also, polytenized *1731* Y sequence has been found in salivary glands (JUNAKOVIC *et al*. 2003). Thus the *1731* in the centromere array may be involved in these activities.

The centromere array has been shaped into clusters resembling the repeated sequences found in the heterochcentromere . As discussed above, the maintenance of *HeT-A* at the Y centromere has been dramatically different from the maintenance of *HeT-A* at telomeres. As a result the ancestral telomere has given rise to two different types of clustered repeats, the more uniform 18HT satellite and a complex set of repeats derived from amplifications of several regions of internally deleted elements. Both types of repeats are head-to-tail arrays, rather than the palindromes which are abundant in protein coding regions on human and chimpanzee Y chromosomes (HUGHES *et al*. 2010). Both types of repeats are also similar to repeated sequences that characterize the heterochromatic centromere regions in chromosomes of multicellular organisms (PERTILE *et al.* 2009; SCHUELER and SULLIVAN 2006; SULLIVAN *et al*. 2001; SUN *et al*. 2003).

Because centromeres in multicellular organisms are determined epigenetically (MALIK and HENIKOFF 2009; SULLIVAN *et al*. 2001), it is not possible to identify centromeres by sequence alone. Nevertheless, this Y cluster is similar in size, repeated sequence structure, and presence of transposable elements to the only functionally characterized centromere in Drosophila, the X chromosome centromere (SUN *et al*. 2003). The similarities between the Y cluster and the X centromere do not extend to the

level of the nucleotide sequence. Because the Y chromosome is the one chromosome which does not pair normally with its homologue, it is not surprising that the Y does not share centromeric sequences with the homologue. Y-specific centromere sequences could well be either a cause or an effect of this non-pairing behavior. [The mouse Y centromere is a known example of Y-specific centromere sequences (PERTILE *et al*. 2009)]

There are reports that some characteristics of replication and/or repair in heterochromatin differ from those in euchromatin (ANDERSON *et al*. 2008; PENG and KARPEN 2008). We suggest that the structure of these *HeT-A*-derived clusters might be in part determined by mechanisms preferentially used in pericentric regions, in addition to being driven by selection for function. For instance, repeated amplification of portions of this sequence is an effective way of providing the rapid evolution that has been noted for centromere regions (HENIKOFF and MALIK 2002).

The strong conservation of the 3'-most end of *HeT-A* in both 18HT and the more complex repeats in the Y centromeric complex may also be driven by function. There is growing evidence that RNA transcripts may be important for the formation of heterochromatin and for some aspects of centromere function (LEE 2009; MALIK and HENIKOFF 2009; SAVITSKY *et al*. 2006; SLOTKIN and MARTIENSSEN 2007). Start sites for both the sense and the antisense transcripts of *HeT-A* lie in the conserved 3' region; sense strand start sites are found 93, 62, and 31 nt upstream of the 3'oligoA (DANILEVSKAYA *et al*. 1997; MAXWELL *et al*. 2006), while multiple antisense starts have been found 220 to 190 nt from the oligoA (SHPIZ *et al*. 2009). Thus it is possible that the 3' region is conserved to direct transcription from this sequence. Northern blots

show several transcripts <1 kb in length with sequence homology to the 3' region (DANILEVSKAYA *et al*. 1999). Some of these RNAs are found only in males and might be products of the Y centromere. However we have also found 3' fragments of *HeT-A* in several intergenic regions on the Y so the origin of the transcripts will require further study.

METHODS

Sequences analyzed

The BAC containing telomere sequence from the 4R telomere is AC010841. The XL telomere sequence is CP000372, a scaffold made by directed finishing of sequence including the most distal gene on XL, *CG17636*, and extending some 20 kb into the telomere array. The BAC containing sequence from the Y centromere region is BACR26J21 (MENDEZ-LAGO *et al*. 2009). The canonical *HeT-A* element is 23Zn-1 (U06920 bp 1015-7097); For Drosophila canonical sequences see http://chervil.bio.indiana.edu:7092/transposons/transposon_sequence_set.em.

Definition of sequence included in *HeT-A*/*TART* telomere arrays

We define *HeT-A*/*TART* telomere arrays as the sequence on the chromosome end that is distal to the most distal *HeT-A* or *TART* element disrupted by insertion of a non-telomere transposable element. The disrupted element marks the end of the transition zone, the proximal end of the telomere where non-telomere elements have invaded *HeT-A* and *TART* sequences. *HeT-A* Tags are recognizable 3'-most sequences of *HeT-A* that are transferred to the 5'end of a downstream element in the course of *HeT-A* transcription. They can form long "strings" on the 5'end of complete elements; each

complete element in these arrays carries a Tag string. A fifth Tag string lies just 5' of the Transition Zone on 4R where its parent element has apparently been invaded by a 1360 transposon.

The most interior Tags in long strings can become too decayed to be recognized and are indistinguishable from the 5'UTR of the element to which they are attached; by default, we include these decayed Tags in the 5'UTR. An exhaustive search for Tags was made during final annotation of the 4R and XL sequences after it had been recognized just how important their role might be in telomere maintenance. Using lowered stringency Blastn, we searched for terminal fragments of *HeT-A's* well-conserved 30 nucleotide 3' terminus in short overlapping regions of the 5' end of each *HeT-A* and the 3' end of its upstream neighbor . (Only searches of short sequences identified all Tags.) Our search protocol ended the search at the last clearly defined Tag found, even if another could be seen within the nearby 5'UTR. This procedure was adopted because short TA(n) or TTA(n), n<5 abound within the AT rich 5'UTR. Also conservatively, because it is likely that they are the result of replication slippage long after they moved into the interior of the telomere, we omit all but the first of several consecutive short sequences that make up the proximal end of the Tag string on XL.

Not unexpectedly, Tag length is not distributed normally. In this paper, we find it most descriptive to report mean lengths and their 95% confidence intervals.

For most calculations we omit elements truncated by cloning at the end of 4R and XL

End Erosion and Terminal Deletion Data Processing.

Analysis was performed using Excel and JMP 8 (SAS Institute, Inc, SAS Campus Drive, Cary, NC, USA, 27513)

Dotplot Analysis

Dotplots comparing the canonical *HeT-A* with the sequences in the BACs were compared by NCBI BLAST (bl2seq) using the default parameters for somewhat similar sequences (blastn). This procedure gives adequate alignment of the canonical sequence with sequences of all known *HeT-A* subfamilies. More stringent BLAST parameters show less sequence in the alignment.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Analysis of *HeT-A*-related sequences in the 4R telomere array. The most distal 76 kb of sequence on the 4R telomere is compared with the sequence of the canonical 6 kb complete *HeT-A* (23Zn-1) by the least stringent NCBI BLAST algorithm (blastn) to ensure alignment with the several subfamilies of *HeT-A* elements in this

array. Sequence of the 76 kb of sequence assembled from the telomere on this chromosome end is shown on the X-axis with the distal end on the left. The *HeT-A* sequence is diagrammed on the Y-axis, showing 5'UTR, ORF, and 3'UTR. The dotted line near the right end defines the 6.2 kb transition zone (T.Z.) where elements from the euchromatic and subtelomeric regions have invaded the telomeric array. For clarity only *HeT-A* sequences are shown on the plot. All *HeT-A* elements, complete and partial, touch the top line because they have complete 3'ends. Arrows on top line mark the eight partial *HeT-A*s. Asterisks indicate partial elements with less than full-length 5'UTRs; these two elements still may be replicatively competent. Arrows at bottom mark the three complete *HeT-A*s. The empty space on the left is occupied by two complete and two partial *TART*s. A very short partial *TART* lies just after the most proximal full length *HeT-A*. Empty space in the T.Z. is occupied by fragments of non-telomeric elements and also *TART* fragments.

Figure 2. HeT Tags in telomere arrays on 4R and XL. Tag length includes oligoA. **(A)** Histogram of Tag length. The 35 individual Tags in this study are grouped by size. **(B)** Organization of Tags into strings. Within each string individual Tags are ordered from distal to proximal with distal on the left. Each cluster is labeled by the FlyBase identifier of its associated HeT-A element (see chromosomes X and 4 at http://flybase.org/cgi-bin/gbrowse/dmel/). Cluster 4R-{4617} lies at the border of the Transition Zone without an associated complete HeT-A. but contributes so much statistically typical data that it is included for Tag analysis. It is identified by the immediately 5' partial HeT-A element, HeT-A{4617} which is the first element in the transition zone.

Figure 3. Complete and 5'-truncated *HeT-A*s in telomere arrays on 4R and XL rank ordered by length. Elements are named by their position (distal-to-proximal) in their telomere array. Light gray bars; 5-truncated elements. Black bars: intact elements (with Tag clusters removed).

Figure 4. (A) Analysis of *HeT-A*-related sequences in the *HeT-A* array in the centromeric region of the Y chromosome. Sequence of 76 kb of the cluster in the sequenced BAC is compared with the sequence of the canonical 6 kb complete *HeT-A* exactly as the 4R telomere sequence was compared in Figure 1. The *HeT-A* sequence is diagrammed on the Y axis, showing 5'UTR, ORF, and 3'UTR. The dotted line near the left end of the plot marks the division between the sequence amplified in the 18HT satellite (first few repeats shown on the left) and the sequence of the ancestral complete *HeT-A* elements (on the right). These *HeT-A*s gave rise to eleven elements but the 3'end of the last was cut off by cloning. Eleven 5' ends can be detected as small fragments reaching the bottom line and the ten 3' ends as small fragments reaching the top line. These fragments are more easily seen in the higher magnification of (B). Gaps in the elements indicate significant loss of sequence. The horizontal gaps contain sequence of non-telomeric transposable elements. **(B)** Comparisons of representative elements from the centromeric *HeT-A* array with the canonical *HeT-A*. Dotplots of individual elements were made as in (A) except that sequence of invading transposable elements was removed from each element. Note that here the *HeT-A* element is shown on the X-axis and the centromere elements are on the Y-axis. For description and order

of elements see Table 1. Element A now has three transposable elements inserted. Their sequence was removed before the dotplot but some unidentified sequence remains. This element shows more sequence rearrangement than the others. There are two inversions (lines of opposite slant) and one of the inverted fragments is moved out of colinear position. Element E was derived by amplification of A (Mendez-Lago, et al., 2009) but both elements have undergone changes since the amplification. They now have 68% identity. Element H gave rise to J. These elements now have 99.7% identity. Element I gave rise to K, the element that was 3'-truncated by cloning. These elements have 98.9% identity where they overlap. These elements are representative of the set. All have segments identical to the extreme 5' and 3' ends of *HeT-A* (lines touching the lower left and upper right corners) but some are difficult to see at this magnification.

TABLE

Element	*Length*	*Longest ORF*
A *(6)*	2139	510
B *(7,6)*	1668	558
C *(7,6)*	1887	558
D *(7)*	2854	447
E *(6)*	1884	558
F *(7)*	3178	318
G *(8)*	3640	528
H *(9)*	3192	381
I *(9,8)*	2567	246
J *(9)*	3185	381
K *(9,8)*	1609	246

Table 1. Elements in the Y chromosomal *HeT-A* array. Elements are designated by single letters in their order on the BAC. Numbers in parenthesis following the letter show the ancestral elements identified by MENDEZ-LAGO *et al*. (2009) as contributing sequence to that element. Any sequence from other transposable elements was removed before analysis of these elements. Length of the element is given in nucleotides including both 5' tags and 3'oligoA. Longest ORF shows number of nucleotides currently in the longest open reading frame. Element K was truncated at the 3'end by cloning. For comparison, the mean length of complete *HeT-A* elements in

telomere arrays is 5927 ±104 (S.D.) bp and the mean length of intact *HeT-A* ORFs is 2821 ± 41 (S.D.) bp.

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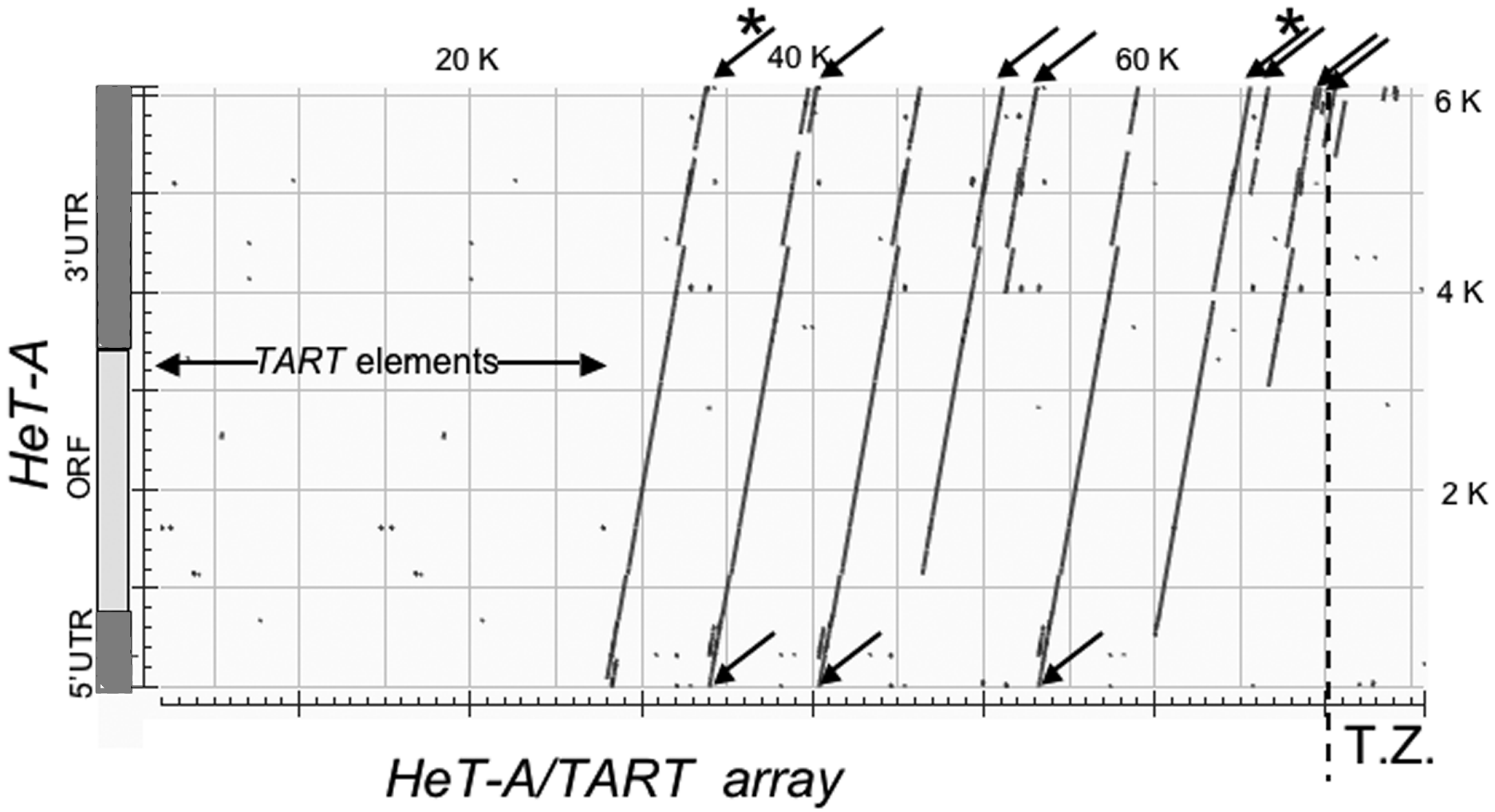
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*
*
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ORF
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TART elements
T.Z.
HeT-A/TART array

Fig.1

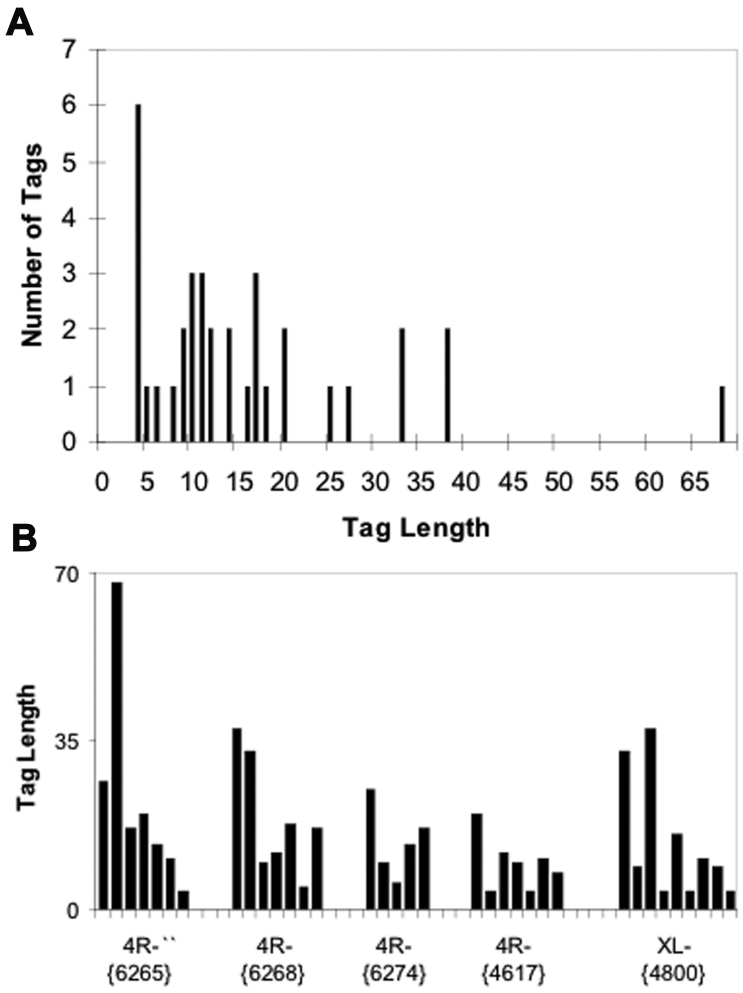
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Fig. 2

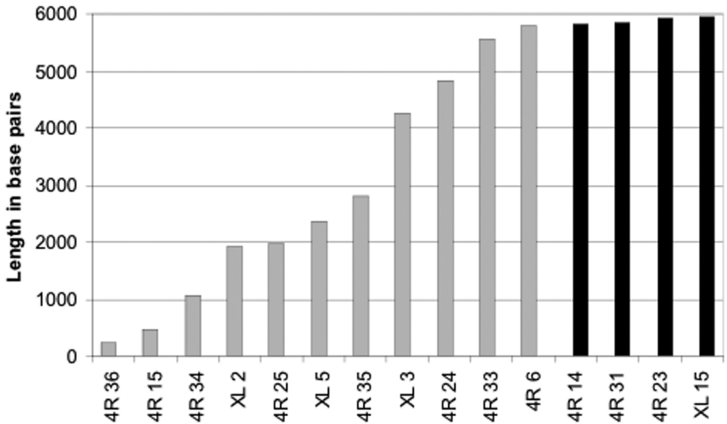
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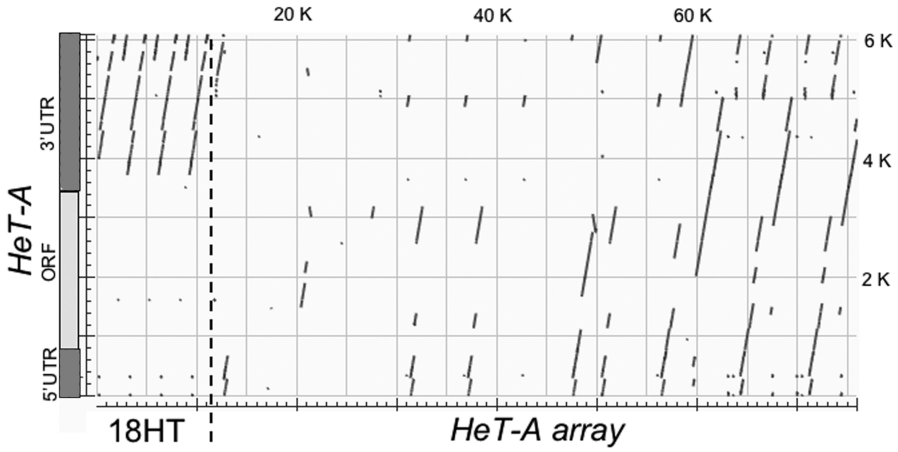
Fig. 3

A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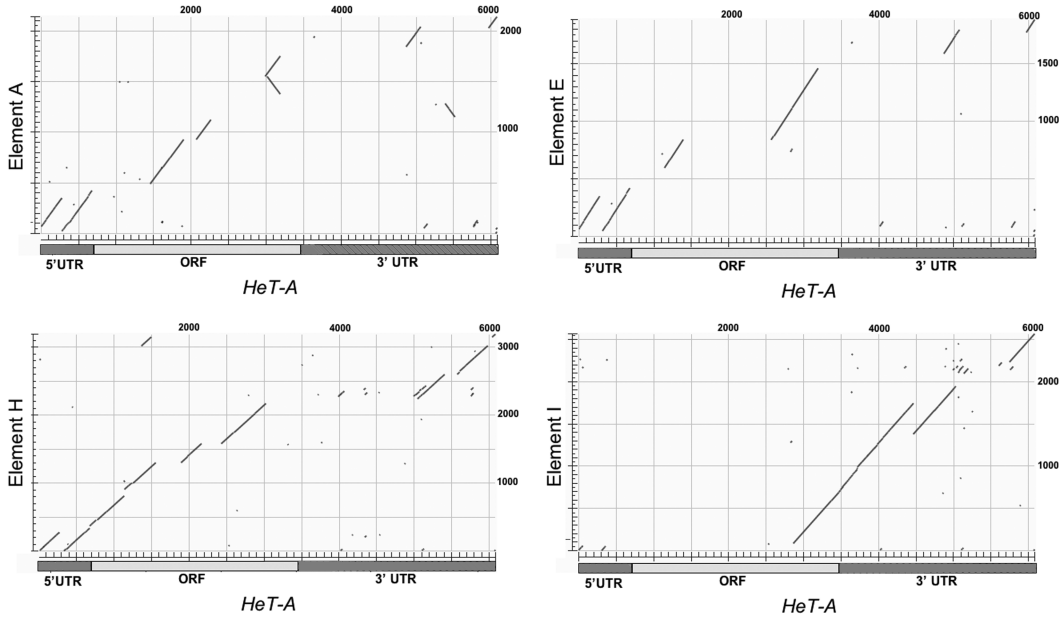
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ORF
3' UTR
HeT-A

Fig. 4