What happens when *Penelope* comes?

An unusual retroelement invades a host species genome exploring different strategies

Michael B. Evgen'ev[1,2]

[1]Engelhardt Institute of Molecular Biology; Russian Academy of Sciences; Moscow, Russia; [2]Institute of Cell Biophysics; Russian Academy of Sciences; Moscow, Russia

Transposable elements (TEs) are ubiquitous residents in eukaryotic genomes. They can cause dramatic changes in gene expression and lead to gross rearrangements of chromosome structure, providing the basis for rapid evolution. The *virilis* species group of *Drosophila* contains certain species that can be crossed under experimental conditions and their phylogeny is thoroughly investigated. We have shown that *Drosophila virilis,* the most primitive karyotypically and probably the ancestral species of the group, is in the process of colonization by a very unusual retroelement *Penelope* which apparently repeatedly invaded the species of the group in the past. However, the molecular mechanisms and evolutionary consequences of such invasions are poorly understood. In this commentary, we discuss the implications of our recent investigation into the response of the RNA silencing system to *Penelope* invasion of a new host genome which can be achieved in different ways.

The *virilis* Species Group of *Drosophila* and *Penelope* Retroelement

We recently reported the results of experiments that explored a possibility to introduce a potentially mobile copy of the *Penelope* retroelement into the genomes of two distant *Drosophila* species.[1] This investigation represents an important step in our long-term studies of *D. virilis* transposons and their possible role in evolution of closely related species belonging to the "virilis" group,[2-4] with special emphasis on *Penelope* which was previously implicated in hybrid dysgenesis (HD) syndrome in *D. virilis*.[5,6] In *Drosophila*, *Penelope* has only been found in the *virilis* group and in *D. willistoni*, however, these TEs termed PLEs (*Penelope*-like elements) in recent years were described in many organisms including fishes, reptiles and rotifers.[7-9] PLEs characteristically differ from the other groups of retroelements by the presence of GIY-YIG-endonuclease domain and an ability to retain their introns in the course of proliferation.[7] *Penelope*-like elements (PLEs) represent an ancient enigmatic superfamily of retroelements that apparently shares a common ancestor with telomerase reverse transcriptases.[10] It is of note that *Penelope* endonuclease domain was first described in group I mobile introns from bacteria and organelles.[7]

Penelope family in *D. virilis* and related species is represented by highly variable structure of individual copies.[5,11] **Figure 1A** depicts a typical structure of *Penelope* element "unit" successfully used in developing transgenic strains[1] which contains 2.8 kb sequence flanked by *XhoI* sites and apparently all necessary elements for expression and transposition in the genome of host species.

The *virilis* group comprises 12 species that are traditionally divided into two phylads: the *D. virilis* phylad and the *D. montana* phylad.[12] In the course of thorough phylogenetic analysis three divergent clades of *Penelope* were detected in the species of the group.[11,13] Importantly, divergence times of the *Penelope* elements found in certain species were smaller than the age of the species, suggesting that

horizontal transfer and multiple invasions by this TE took place in the course of the *virilis* group species evolution.[13]

In our model system we have a unique opportunity to investigate the behavior of this retroelement in two distant species, i.e., *Drosophila virilis* and *Drosophila melanogaster* separated by 50–60 million years of divergent evolution.[12] While various clades of *Penelope* were found in *D. virilis* strains,[11,13] no trace of *Penelope* was detected in the sequenced *D. melanogaster* genome[14] which is naïve in terms of *Penelope* presence.

Penelope elements can invade and amplify in a new host genome in a number of different ways, as described below.

Invasion of *D. virils* natural populations. Transposons can be transmitted horizontally and spread through interbreeding.[15] However, there are multiple mechanisms underlying TE silencing and limiting the invasion process.[16-18] Piwiclade Argonaute proteins were shown to have a prominent role in transposon silencing in vivo in various plants and animals.[19,20] Mechanisms of piRNAs biogenesis are best understood in *D. melanogaster* where they arise from TEs that have landed within certain genomic loci designated as piRNA clusters.[20,21] The TEs are controlled in the germ line by short, antisense, TE-derived RNAs (23–29 nt) that are found in complexes with three Piwi-clade Argonaute proteins.[21] The piRNA clusters drive the production of primary piRNAs that prime a larger pool of secondary piRNAs through repeated cycles ("ping-pong amplification loop") of destruction of sense and antisense TE transcripts.[20,21]

As we mentioned above basing on phylogenetic analysis, various species of the *virilis* group have been colonized by *Penelope* at different stages of their divergent evolution.[11,13] Previously, we provided substantial evidence that cosmopolitan species *D. virilis* is at the present time in the process of colonization by *Penelope* family of retroelements. We were lucky to directly demonstrate recent *Penelope* invasion of a natural *D. virilis* population (Middle Asia, Tashkent).[2] Thus, the *D. virilis* strains collected in this area in 1968 were free of *Penelope* sequences, while all individuals collected from the

same population in 1997 carried multiple *Penelope* copies located exclusively in euchromatic chromosomes arms.[2] In situ analysis exploring *Penelope* probe detected asymmetrical hybridization frequently observed in unpaired regions of polytene chromosomes in the progeny of freshly caught flies in the 1997 population, indicating that an exceptionally high level of heterozygosity was present in the contemporary Tashkent population.[2] Interestingly, the ongoing invasion of *Penelope* occurs in *D. virilis*, a cosmopolitan species which is itself in the process of global demographic expansion, probably related to human movements.[22] Careful analysis of multiple laboratory and geographical strains of *D. virilis* demonstrated different content of full-length (**Fig. 1A**) and potentially functional copies of *Penelope* elements.[1-3,5] Most of the studied *D. virilis* geographical strains including Tashkent strain recently invaded by this TE exhibit neutral cytotypes i.e., do not exhibit high level of gonadal sterility when crossed with tester strains.[1,5,6] Therefore, apparently the appearance of *Penelope* elements may rapidly change the M cytotype characteristic of original *Penelope*-free strains to neutral status. Furthermore, practically all *Penelope*-containing strains are characterized by the presence of *Penelope*-derived piRNAs in their ovaries[1] and these piRNAs are faithfully transmitted to the progeny (**Fig. 1B**). It is of note that, among the analyzed strains, there are several exceptional ones which exhibit a neutral cytotype but seem not to contain *Penelope* sequences and *Penelope*-homologous piRNAs. However, some of these strains do contain defective *Penelope* copies. In summary, large scale analysis of *D. virilis* strains enables us to conclude that natural invasion of *Penelope* leads to rapid change of strain cytotype, which usually correlates with the presence of *Penelope*-derived piRNAs in the ovaries.[1]

Penelope* transpositions due to dysgenic crosses in *D. virilis. Dysgenic crosses between certain strains of *D. virilis* differing by the presence of full-length copies of this element represent another approach enabling rapid transposition and amplification of *Penelope* sequences in the genome.[5,6] Contrary to the previously

described syndromes in *D. melanogaster* (*P-M* and *I-R* syndromes), where *P* and *I* elements are activated independently in different systems,[23,24] the HD syndrome in *D. virilis,* which includes male and female gonadal sterility, multiple point and chromosomal mutations and other abnormalities observed in the progeny of dysgenic cross, probably results from simultaneous activation of several unrelated TEs.[5,6,25] It was shown that besides *Penelope*, retroelements *Ulysses, Helena* and *Telemac*, as well as DNA transposon *Paris,* are also mobilized and may cause mutations in the same dysgenic cross.[25]

Recently with the development of deep-sequencing techniques it was clearly demonstrated that *P-M* hybrid dysgenesis in *D. melanogaster* also activates both *P*-elements and other resident transposons and disrupts the piRNA biogenesis machinery.[26] Furthermore, interspecific hybrids between *D. melanogaster* and *D. simulans* are characterized by widespread derepression of multiple both maternally and paternally inherited TE families.[27] It is of note that in the early eighties, soon after the discovery of mobile elements in *Drosophila*, we were the first to directly demonstrate transposition of a family of dispersed mobile repeats ("*pDv* elements") in interspecific hybrids between *D. virilis* and two other species of the *virilis* group (i.e., *D. lummei* and *D. littoralis*).[28]

Previous studies suggested that a key driver in *D. virilis* HD syndrome is the *Penelope* retroelement, which was proposed not only to become mobile itself, but also to mobilize other TEs mentioned above in the dysgenic hybrids.[5,6] It was suggested that *Penelope* expression somehow interferes with RNAi machinery involved in silencing of other unrelated TEs.[29] Although recent reports have implicated RNA silencing in repression of hybrid dysgenesis in *D. virilis*[29] and in *D. melanogaster*,[26,30,31] in *D. virilis* the evidence in favor of direct and critical role of *Penelope*-homologous small RNAs in HD syndrome is not so straightforward.[29,32] In the first report on the role of *Penelope*-derived small RNAs in the HD the authors did not discriminate between *Penelope*-derived siRNAs and piRNAs and, hence, encountered some difficulties in localization of the "master
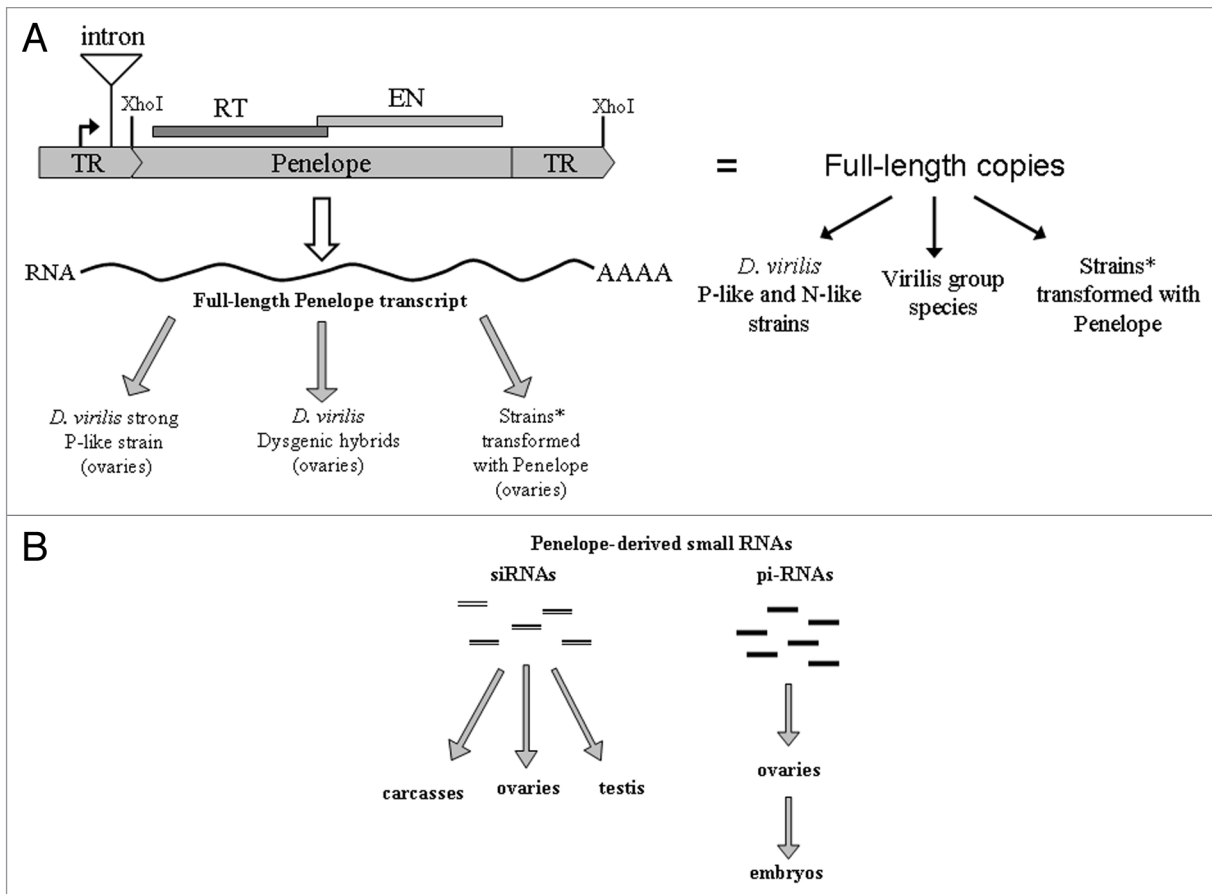
Figure 1. The distribution of full-length *Penelope* copies and *Penelope*-derived transcripts in various strains and species of *Drosophila*. (**A**) The presence of intact and potentially active *Penelope* copies and canonical (2.8 kb) *Penelope* transcripts in different *Drosophila* strains and species. The position of intron is indicated. Black arrow indicates the transcription start. *In our experiments we obtained strains transformed with *Penelope* using both *D. virilis* and *D. melanogaster*. TR-terminal repeats which can be in tandem or inverse orientation in different *Penelope* copies. RT-reverse transcriptase; EN-endonuclease. Cleavage sites of *XhoI* endonuclease are indicated. (**B**) *Penelope*-homologous small RNAs detected in the *virilis* group species, certain *D. virilis* strains and a few strains transformed with *Penelope*. Maternally transmitted *Penelope*-derived piRNAs may target *Penelope* mRNA through transcript cleavage.

locus" producing *Penelope*-derived small RNAs.[29] Furthermore, although in a subsequent investigation we did observe correlation between *Penelope* transcription in the ovaries of dysgenic hybrids and manifestation of dysgenic traits, we could not always correlate differences in maternally deposited *Penelope* piRNA in the hybrids between various *D. virilis* strains with the sterility of the progeny.[32] Our investigation also demonstrated that *Penelope* transposes with low frequency in *D. virilis* strain 160 used as a *P*-like in the dysgenic crosses.[33] It was shown that small RNAs homologous to *Penelope* found in this particular strain, belong predominantly to the siRNA category (**Fig. 2**), and consist of sense and antisense species observed in approximately equal proportion (**Fig. 3**). The number of *Penelope* copies in the

latter strain has significantly increased during the last decades, probably because *Penelope*-derived siRNAs are not maternally inherited, while the low level of *Penelope*-piRNAs, which are transmitted from mother to the embryo, is not sufficient to silence this element completely in strain 160. Therefore, we speculated that intrastrain transposition of *Penelope* is controlled predominantly at the posttranscriptional level.[33]

High transposition frequency of various TEs induced by dysgenic crosses may play an important role in evolution of the *virilis* species group. Some time ago, we observed multiple chromosomal rearrangements in the progeny of dysgenic crosses between certain *D. virilis* strains.[3] Remarkably, many rearrangement breakpoints at cytological level coincide with

the chromosomal locations of *Penelope* and *Ulysses* insertions in the parental strains and with breakpoints of inversions previously established for other species of the group.[3]

Further analysis revealed the presence of full-length-*Penelope* copies and *Penelope*-derived piRNAs in the ovaries of practically all species of the *virilis* group (**Fig. 1A**), which, however, correlates with apparent transcriptional inactivation of *Penelope* in all the species with the exception of *D. virilis*.[1]

Direct introduction of *Penelope* into the genomes of *D. virilis* and *D. melanogaster*. Fortunately, in our model system we could explore a unique opportunity to experimentally imitate evolution and investigate the behavior of this unusual retroelement directly introduced into the
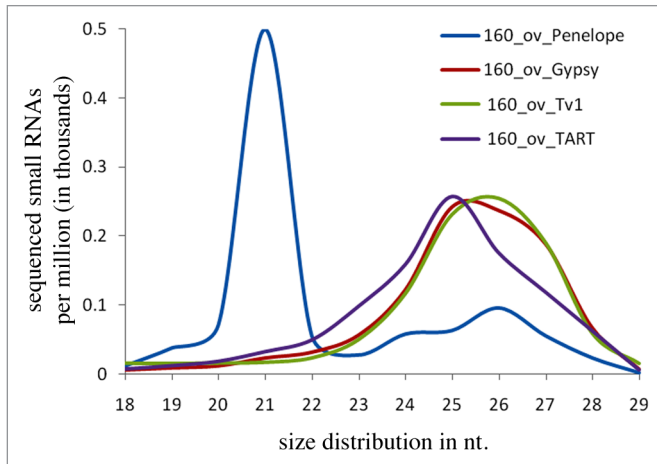
Figure 2. Size profile (in nt) of small RNAs derived from *Penelope, Gypsy, Tv1* and *TART* retroelements[32] in the ovaries of *D. virilis P*-like strain 160.
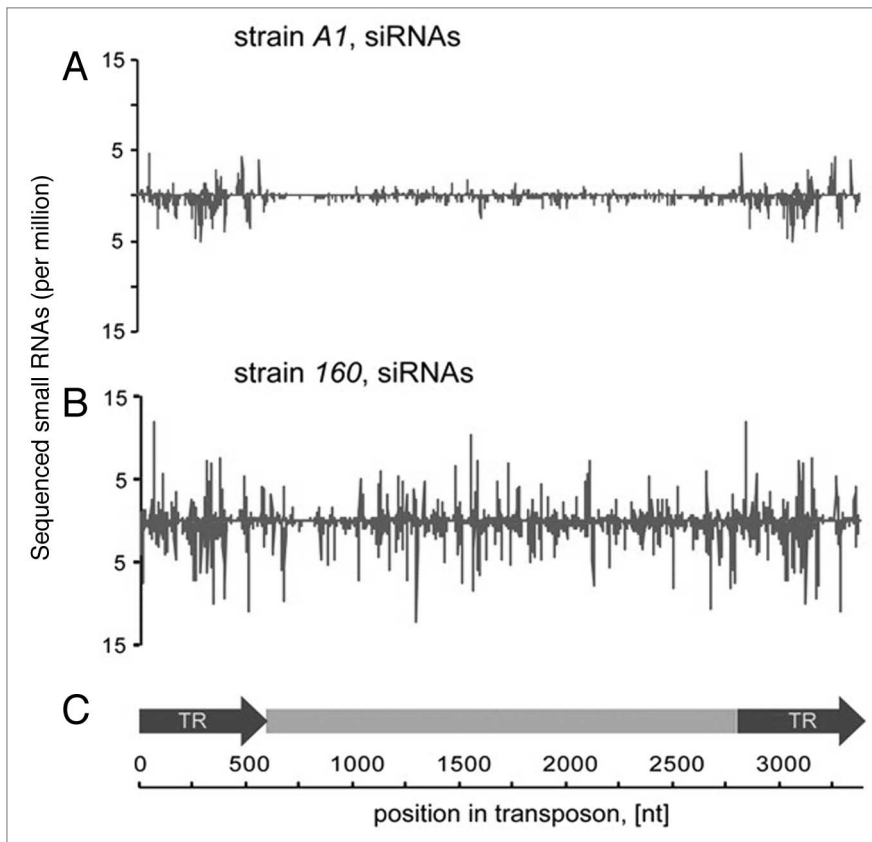

Figure 3. Distribution of *Penelope*-derived siRNA (21–23 nt) along the transposon body in *D. melanogaster* strain (A1) transformed by *Penelope* (**A**) and in *D. virilis* strain 160 (**B**). The structure of the consensus *Penelope* element, containing two terminal repeats used in transformation experiments is shown at the bottom of the figure. The figure is adapted from reference 32.

strains of two distantly related species, *D. virilis* and *D. melanogaster*. The recipient strain of *D. melanogaster* was *Penelope*-free, while the *D. virilis* strain used in the transformation experiments contains only inactive heterochromatic copies of this retroelement (clade II), probably representing the remnants of previous ancient invasions.[1,11,13] More than a decade ago we introduced full-length *Penelope* copies (**Fig. 1A**) into the *D. melanogaster* genome exploring *P*-mediated transformation.[34] The introduced copies were actively transcribed and eventually amplified in the *D. melanogaster* genome.[34] Three years ago we investigated the pattern of *Penelope*-derived small RNAs in the strains transformed by *Penelope*. The analysis demonstrated the presence of *Penelope*-derived siRNAs (21–22 nt) in the carcasses of the transgenic strains studied.[32] The mechanisms by which TEs become recognized by the siRNA pathway are not fully understood, however, previously in the transgenic strains we described multiple rearranged *Penelope* copies containing long inverted repeats which could give rise to double-stranded RNAs (dsRNAs) that are processed into siRNAs.[34] Remarkably, in *D. melanogaster* transformed strains the detected siRNAs precisely correspond to these inverted regions within *Penelope* body.[32] On the other hand, in the *D. virilis* strain 160 *Penelope*-homologous siRNAs were randomly distributed along the *Penelope* body and probably result from bidirectional transcription of the TE (**Fig. 3A and B**). At the next step we performed broad scale analysis of multiple (35 total) iso-female derivatives of several *D. melanogaster* strains transformed with *Penelope* in 2001. While in the carcasses of these strains *Penelope*-derived siRNAs (21nt) were frequently seen, in the ovaries of a few strains we detected by Northern hybridization 25–27 nt small RNAs homologous to the *Penelope* transcript that were subsequently shown to belong to the piRNA category[1] (**Fig. 1B**).

The appearance of piRNAs in the ovaries of transgenic strains probably resulted from accidental transposition of *Penelope* copy into one of the piRNA genomic clusters of *D. melanogaster*. To prove our assumption, we determined the localization of *Penelope* inserts in the genomes of derivative transgenic iso-female strains characterized by the presence of piRNA in the gonads. Interestingly, in two of these strains we detected *Penelope* insertion within the major *D. melanogaster* germ line-specific piRNA 42AB cluster.[20] It was also shown that *Penelope* expression is severely inhibited in the transgenic strains containing *Penelope*-homologous piRNAs in the ovaries.[1]

Furthermore, we crossed the strains containing *Penelope*-derived piRNAs in the ovaries with the strains lacking *Penelope* and provided evidence that detected piRNAs are maternally deposited and can silence euchromatic transcriptionally active copies of *Penelope in trans*. Such Trans-Silencing Effect is described for the X-TAS system and *P*-element insertions in telomeric regions.[35]

To further elucidate the fate of *Penelope* in terms of its expression and RNA biogenesis at the early stage of reinvasion in the *D. virilis* genome, we imitated the phenomenon by introducing full-size *Penelope* copies into a typical M-like strain of *D. virilis* devoid of functional copies of this transposon[5,11] using the *piggy-Bac*-based transgenesis system.[36] We established several independent transgenic strains containing inserts of a full-length *Penelope* (**Fig. 1A**). In situ hybridization experiments demonstrated, besides the sites of the original *white*-containing constructs, additional sites of *Penelope* insertion in the chromosomes of the transformed strains. Northern blotting and RT-PCR technique revealed a significant level of *Penelope* transcription in all obtained transgenic *D. virilis* strains, both in the ovaries and in the carcasses.[1] A detailed cytological analysis of *Penelope* localization in the transgenic strains demonstrated that, in contrast to the original construct insertion loci that are present in all nuclei of the salivary glands and serve as an internal control, additional hybridization sites are usually seen in only a fraction of salivary gland nuclei and, hence, transgenic larvae represent mosaics in terms of *Penelope* presence. Therefore, in these experiments we demonstrated that initial transposition of *Penelope* after its introduction takes place predominantly in somatic tissues.[1] Interestingly, a decade ago, when several *D. melanogaster* strains transformed with the same transposon were developed, we also often observed similar mosaics in salivary gland nuclei immediately after transformation by in situ hybridization

and single fly Southern blot hybridization analysis of the transformed strains performed at that time.[1] It is of note that multiple somatic transpositions were previously reported for various non-LTR retroelements.[37]

Conclusions

We explored a unique chance to monitor the consequences of a very unusual retroelement *Penelope* introduction into the genomes of *D. virilis* and *D. melanogaster*. We compared the outcome of natural invasion of *Penelope* into *D. virilis* species, the consequences of dysgenic crosses, and the results of direct introduction of this TE into the genomes of the two species by transformation with full-length *Penelope*. At the initial stages of transposition *Penelope* tends to produce rearranged copies, which may give rise to siRNAs from the inverted repeats. These *Penelope*-derived siRNA are not able to efficiently silence *Penelope,* which leads to active somatic transpositions and *Penelope* amplification in the genome of a new host species. However, with time *Penelope* copies can find their way to pi-clusters and become transcriptionally silenced by *Penelope*-homologous piRNAs. The colonization of host genomes by *Penelope* may rapidly change the cytotype and induce gross rearrangements of the chromosomes providing the basis for rapid evolution.

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