Mobile elements and chromosomal evolution in the *virilis* group of *Drosophila*

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Species of the *virilis* group of *Drosophila* differ by multiple inversions and chromosome fusions that probably accompanied, or led to, speciation. *Drosophila virilis* has the primitive karyotype for the group, and natural populations are exceptional in having no chromosomal polymorphisms. We report that the genomic locations of *Penelope* and *Ulysses* transposons are nonrandomly distributed in 12 strains of *D. virilis*. Furthermore, *Penelope* and *Ulysses* insertion sites in *D. virilis* show a statistically significant association with the breakpoints of inversions found in other species of the *virilis* group. Sixteen newly induced chromosomal rearrangements were isolated from the progeny of *D. virilis* hybrid dysgenic crosses, including 12 inversions, 2 translocations, and 2 deletions. *Penelope* and *Ulysses* were associated with the breakpoints of over half of these new rearrangements. Many rearrangement breakpoints also 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. Analysis of homologous sequences from *D. virilis* and *Drosophila lummei* indicated that *Penelope* insertion sites were closely, but not identically, located at the nucleotide sequence level. Overall, these results indicate that *Penelope* and *Ulysses* insert in a limited number of genomic locations and are consistent with the possibility that these elements play an important role in the evolution of the *virilis* species group.

Besides inducing many types of small mutations, such as insertions and deletions, transposable elements (TEs) are well known to promote the formation of inversions and other large and small chromosomal rearrangements (e.g., ref. 1). A growing body of evidence suggests that TEs mediate genome restructuring in natural populations of a wide variety of species. For example, in hominids, a *Y* chromosome inversion was mediated by recombination between *LINE-1* elements before the radiation of extant human populations (2), and TEs appear to have played a role in mediating some of the major restructuring of the human sex chromosomes that has taken place during the last 300 million years (3). Also, the five families of *Ty* retrotransposons have been important in restructuring the *Saccharomyces cerevisiae* genome (4). In wild populations of *Drosophila melanogaster*, the *hobo* element has been implicated in the origin of endemic inversions (5) and in *Drosophila buzzatii,* the breakpoints (BPs) of a cosmopolitan inversion contain large insertions corresponding to a TE (6). The frequency and relative importance of TE-induced rearrangements in natural populations have, however, been difficult to establish in any satisfactory quantitative way. One likely reason is that instability and rapid divergence of TEs make their identification at rearrangement BPs increasingly difficult with the passage of time (7).

In contrast to the rich karyotypic variation found in most of the 12 species of the *D. virilis* species group, one member, *D. virilis*, is exceptional in having a monomorphic, relatively primitive, karyotype (8). No spontaneous inversions have previously been reported in geographical strains of *D. virilis*, despite extensive searches (9–12).

Contrary to the normal mode of independent activation, at least five structurally different families of TEs are simultaneously activated by interstrain hybridization (hybrid dysgenesis) in *D. virilis*. These families include *Ulysses, Helena, Paris*, *Telemac*, and *Penelope* (13–15). The *Penelope* family of elements has been identified as playing a critical role in *D. virilis* hybrid dysgenesis (15, 16). This family is also exceptional in being absent from a number of *D. virilis* strains but is present and active in others. This patchy distribution has been explained by a recent *Penelope* invasion of the species (17).

Full-sized and at least potentially functional copies of *Penelope* and *Ulysses* were previously found in most species of the *virilis* group (18). Exceptionally, the *Penelope* element was restricted to the chromocenter in *Drosophila kanekoi, Drosophila lummei,* and some strains of *D. virilis* (15), suggesting the presence of only inactive elements. The chromosomal distributions of *Penelope* and *Ulysses* in other species of the *virilis* group showed highly significant deviations from independence (18). Surprisingly, a statistically significant coincidence was also observed earlier between the chromosomal subsections in which these elements were inserted and the subsections that contained inversion BPs previously established in species of the *virilis* group (18). Both insertion site preference and selection might have contributed to these nonrandom distribution patterns.

We here report the occurrence of multiple chromosomal rearrangements in the progeny of *Penelope*-activated hybrid dysgenic crosses in *D. virilis*. There is strong statistical support for nonrandom distribution of the insertion sites of *Penelope* and *Ulysses* throughout the *D. virilis* genome. These insertion sites are coincident both with hot spots for BPs of new hybrid dysgenesis-induced rearrangements and BPs of established inversions in other species of the *virilis* group.

Materials and Methods

***Drosophila* Strains.** *D. virilis* strains 1051.51 (Chile), 1051.49 (Argentina), 1051.48 (Mexico), 1051.47 (China), 1051.8 (Truckee, CA), and 1051.9 (Sendai, Japan) were obtained from the *Drosophila* Species Center, Bowling Green, OH.

D. virilis strains 2003 (Uman, Ukraine); 2005 (Magarach, Crimea); 9 (Batumi, Georgia); 160 (*b, gp, cd, pe, gl*); 110 (*tb, gp; st*); 140 (*va, eb)*; and 142 (*st, es*) were obtained from the Stock Center of the Institute of Developmental Biology, Moscow.

Flies stocks were maintained on standard medium at 25°C.

Cytological Analysis. Larvae were grown at 18°C in cultures supplemented with live yeast solution 2 days before dissection. Salivary glands from third instar larvae were dissected in 45%

Abbreviations: TE, transposable element; BP, breakpoint.

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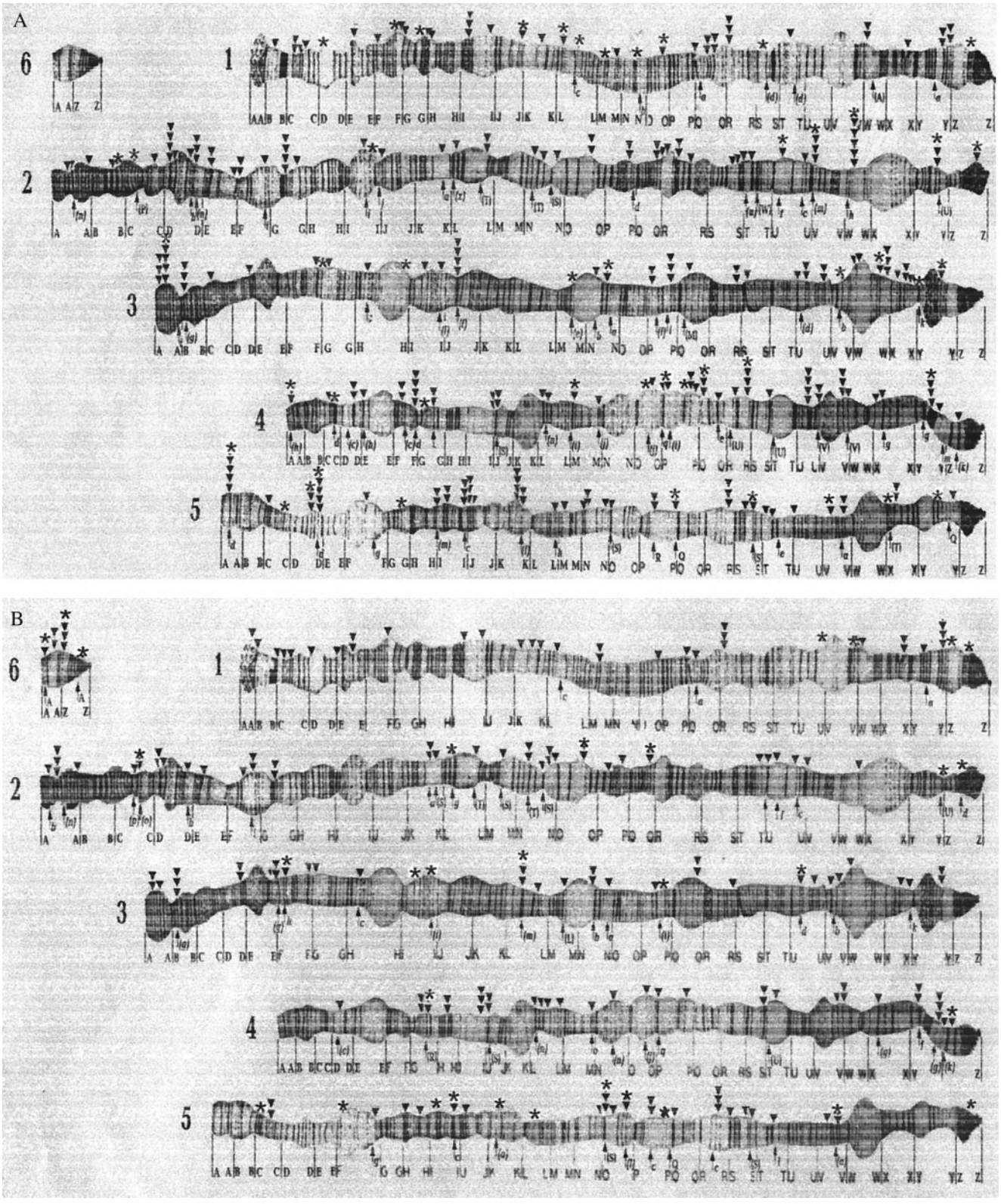

Fig. 1. The distributions of *Penelope* (*A*) and *Ulysses* (*B*) within the *D. virilis* genome as revealed by *in situ* hybridization. The numbers at the left of the figures identify the six chromosomes. The triangles above the chromosomes represent the insertion sites of *Penelope* or *Ulysses* in the 12 *D. virilis* strains studied. The stars above the chromosomes represent the subset of these insertion sites identified in hybrid dysgenesis reference strains 160 and 9 (a total of 47 *Penelope* sites in strain 160 and a total of 32 *Ulysses* sites in strains 160 and 9 combined). The arrows below the chromosomes indicate the positions of inversion BPs previously identified in other species of the *virilis* group. Individual inversions are identified by lowercase letters according to the classification system of refs. 8 and 28. Unique inversions that are polymorphic in individual species of the *virilis* group are shown in parentheses.

Table 1. Distribution of insertion sites, and total numbers of elements inserted (in parentheses), for *Penelope* and *Ulysses*, in the six chromosomes of *D. virilis*

	Number of insertions at a single site							
Chromosome No.	0	1	2	3	4	5	6	Total
Penelope								
1	211 (0)	23 (23)	9 (18)	2 (6)	0	0	0	34 (47)
2	271 (0)	28 (28)	9 (18)	2 (6)	3 (12)	0	1 (6)	43 (70)
3	241 (0)	20 (20)	8 (16)	4 (12)	1 (4)	1 (5)	0	34 (57)
4	208 (0)	20 (20)	10 (20)	2 (6)	4 (16)	0	0	36 (62)
5	227 (0)	17 (17)	10 (20)	4 (12)	1 (4)	2 (10)	0	34 (63)
6	20 (0)	0	0	0	0	0	0	0
Total	1178 (0)	108 (108)	46 (92)	14 (42)	9 (36)	3 (15)	1 (6)	181 (299)
Ulysses								
1	217 (0)	23 (23)	3 (6)	2 (6)	0	0	0	28 (35)
2	281 (0)	23 (23)	9 (18)	1 (3)	0	0	0	33 (44)
3	246 (0)	19 (19)	8 (16)	2 (6)	0	0	0	29 (41)
4	220 (0)	14 (14)	8 (16)	2 (6)	0	0	0	24 (36)
5	235 (0)	19 (19)	5 (10)	2 (6)	0	0	0	26 (35)
6	16 (0)	1 (1)	2 (4)	0	1 (4)	0	0	4 (9)
Total	1215 (0)	99 (99)	35 (70)	9 (27)	1 (4)	0	0	144 (200)

acetic acid and squashed according to procedures developed by Lim (19). Procedures and DNA probes for *in situ* hybridization studies were as described in ref. 18.

DNA Manipulations. Genomic libraries from *D. virilis* strain 160 and *D. lummei* strain 201 (a strain caught in the Moscow region in 1970) were prepared by partial *Sau*3A digestion with subsequent ligation into the *Bam*HI site of Lambda Dash phage arms (Stratagene). A Sequenase I kit (Stratagene) was used to Sequence *Penelope*-containing clones.

Statistical Methods. For the χ^2 test for goodness of fit of observed TE distributions with those expected from a Poisson distribution, the rare classes with five and six elements were pooled. We used the coefficient of dispersion, defined as the ratio of the variance and the mean of a Poisson distribution (e.g., ref. 20). The Poisson parameter, $\ddot{E}_{obs}$, is computed as the ratio of the observed number of elements to that of chromosomal subsections. For a positive Poisson distribution (truncated by omission of the zero class), we estimated the expected Poisson distribution parameter, $\ddot{E}_{exp}$, by the method described in ref. 21, equations 4.79 and 4.82. We then estimated the effective distribution coefficient, $d_e = \ddot{E}_{obs}/\ddot{E}_{exp}$, and $S_e = Sd_e$, where S is the total number of sites (L.A.Z., unpublished work). The parameters d_e *and* S_e are interpreted as, respectively, the fraction and the effective absolute number of subsections that were potentially available for insertion (S = 1,359 for chromosomal subsections of *D. virilis*). The coincidence of mobile element insertion sites and chromosomal BPs was tested by the statistical method used in ref. 18.

Results

The Chromosomal Distribution of *Penelope* and *Ulysses* in *D. virilis*. We examined the chromosomal locations of *Penelope* and *Ulysses* in 12 *D. virilis* strains previously shown to carry these elements (17). Each of the 181 *Penelope* and 144 *Ulysses* euchromatic insertions identified was localized to one of the 1,359 chromosomal subsections of the *D. virilis* genome (11) (Fig. 1 *A* and *B* and Table 1).

We tested whether the distribution of subsections with 1, 2, 3, etc., *Penelope* or *Ulysses* insertions varied among chromosomes. The heterogeneity among chromosomes was found not to be statistically significant (P = 0.64 and 0.44 for *Penelope* and *Ulysses,* respectively) by using the permutation method (10,000 runs) and Fisher's exact test. Analysis of the pooled chromosomal distributions was, therefore, justified and revealed large deviations from a Poisson distribution. A χ^2 test for goodness of fit gave values of 1,761 and 853 with df = 4 for *Penelope* and *Ulysses,* respectively. The observed number of subsections with no elements (the zero class in Table 1) exceeded that expected from a Poisson distribution. The resulting values for the coefficient of dispersion were much larger than 1 (Table 2), and values of the Poisson parameter were smaller than those expected from a positive Poisson distribution.

In contrast, the data of Table 1 fitted a positive Poisson distribution almost perfectly: The corresponding χ^2 values are 10.6 and 0.45 (P = 0.014 and 0.93, for *Penelope* and *Ulysses*, respectively). However, the combined test (χ^2 = 11.1 with df = 6) was insignificant (P = 0.09). Therefore, an excess in the zero class causes the deviation of the observed distribution of mobile elements. The values of the effective distribution coefficient, d_e, are fairly similar in both families of mobile elements (Table 2). These statistical results suggest that only about one-fifth of the chromosomal subsections are actually available for insertion by these elements. The remaining subsections appear effectively unavailable for insertion. The similarity of the distributions of *Penelope* (or *Ulysses*) among different chromosomes, as well as that between the distributions of the two families themselves, suggests that a common mechanism underlies this phenomenon.

Table 2. Distribution parameters of *Penelope* and *Ulysses* calculated from the data in Table 1

		Poisson parameter			
Family	Coefficient of dispersion	$\ddot{E}_{obs}$	$\ddot{E}_{exp}$	Effective distribution, *de*, %	Effective number of sites, *Se*
Penelope	2.03	0.220	1.10 ± 0.20	19.9	271
Ulysses	1.53	0.147	0.70 ± 0.21	21.1	287

Table 3. Nonrandom occurrence of mobile elements *Penelope* (Pen) and *Ulysses* (Uly) at inversion BP in the genome of *D. virilis*

Comparison type	C_{obs}*	C_{exp}†	Significance, *P*	Coincidence, *P*
Pen vs. BP	80	18.78	$\ll$0.001	0.50
Uly vs. BP	57	14.94	$\ll$0.001	0.33
Pen vs. Uly	82	19.18	$\ll$0.001	0.50
Pen and Uly vs. BP	56	8.51	$\ll$0.001	0.65

*C_{obs} is the observed number of common subsections.
†C_{exp} is the expected number of common subsections, given a total of 1,359 subsections in the *D. virilis* chromosome map. The total number of inversion BPs compared in other species of the *virilis* group was 141, and the numbers of *Penelope* and *Ulysses* insertions sites observed in *D. virilis* were 181 and 144, respectively.

Comparison of Fig. 1 *A* and *B* suggests several "hot spots" for *Penelope* and *Ulysses* insertion in all of the large chromosomes. We failed to find any correlations between these hot spots and known cytogenetic landmarks such as weak points or 5S genes in *D. virilis* chromosomes (12). However, when the locations of *Penelope* and *Ulysses* insertion sites were compared with the BPs of inversions previously described in other species of the *virilis* group (8, 11), statistically significant associations were observed (Table 3). The chromosomal subsections of 80 *Penelope,* and 57 *Ulysses* insertion sites coincide with inversion BPs previously described. Furthermore, *Penelope* and *Ulysses* had 82 insertion sites in common in the *D. virilis* strains examined. Similar results were previously observed for the distributions of *Penelope* and *Ulysses* in other species of the *virilis* group (18).

Chromosomal Rearrangements in the Progeny of Dysgenic Crosses. We made dysgenic crosses between females of *D. virilis* strain 9, which had no active *Penelope* copies, and males of strain 160, which carried multiple *Penelope* copies (18). F_1 hybrid females and males were mated *inter se*, and progeny with obvious morphological abnormalities, such as misshapen wings and abdomens, were selected in the F_2 and F_3 generations. When possible, these individuals were used to propagate lines. Each of these individuals was crossed with five males or females of strain 9, and the salivary glands of at least 10 larval progeny per cross were checked for the presence of chromosomal rearrangements. From the progeny of these phenotypically abnormal flies, we isolated 16 new chromosomal rearrangements, including 12 inversions, 2 translocations, and 2 deletions (Table 4; Fig. 2). Although our method did not allow estimation of the frequency of newly occurring rearrangements, abnormal phenotypes served as useful indicators of transpositional activity and increased the probability of rearrangement detection.

In situ hybridization studies revealed that a significant fraction of the BPs of newly induced rearrangements were associated with *Penelope* or *Ulysses* (Table 4). Of a total of 32 BPs, 16 proximal and 16 distal, 10 were associated with *Penelope* and 4 with *Ulysses.* The expected numbers of TE/BP associations are 1.11 for *Penelope* and 0.75 for *Ulysses,* both showing highly significant deviations from those observed ($P = 3.3 \times 10^{-8}$ and 5.7×10^{-3}, respectively). These 14 associations were divided equally between distal and proximal BPs.

The locations of the BPs of the newly induced rearrangements were also compared with the locations of *Penelope* and *Ulysses* in the 2 parental strains, 160 and 9, in which a total of 47 *Penelope* and 32 *Ulysses* insertions were located (Fig. 1 *A* and *B*, respectively). Eleven rearrangement BPs coincided with *Penelope* sites present in parental strain 160. Of these, 10 retained their *Penelope* elements and 1 (BP 5Ci) no longer retained its element (Table 4, column 4). Four inversion BPs coincided with *Ulysses* sites present in either parental strain (Table 4, column 5), and all four BPs retained their *Ulysses* elements.

Finally we compared the location of the BPs of newly induced

Table 4. Characterization of chromosomal rearrangements induced in the progeny of *D. virilis* dysgenic crosses

Type of rearrangement	Location of distal BP	Location of proximal BP	Coincidence of new rearrangement BPs with *Penelope* in strain 160	Coincidence of new rearrangement BPs with *Ulysses* in strains 160 or 9	Coincidence of new rearrangement BPs with Inversion BPs in other species*
Inversions	XDk	XIe			
	XIa	XTf			XTf (N,T,A)
	XRc	XXa			
	2Bj	2Gd	2Bj		
	2Ij	2Za	2Ij, 2Za		2Ij (Mo, La, Ezo, Lu); 2Za (Mo, La, Li, Ezo, Ka)
	2Si/Ta	2Zc			2Si/Ta (Ezo, Lu); 2Zc (Ka)
	3FeS	3Z		3Fe, 3Z	3Fe (Li); 3Z (N, A, Mo, La)
	3Hk	3Wf*	3Hk, 3Wf		3Hk (Mo, La)
	3Nhi	3Ri			3Nhi (A, N)
	4Mhi	4Sa	4Mhi, 4Sa		
	4Od	4Sa	4Sa		4Od (Mo)
	5Iab	5Pn		5Iab, 5Pn	5Pn (Ezo, Ka)
Translocations	XHki	5Ma			
	2Eh	4Ga	2Eh, 4Ga		4Ga (Li)
Deletions	5Ab	5Ci	(5Ci)		
	2Eh	2Fa			
Total observed	16	16	10	4	12
Total expected			1.11	0.75	3.29
Significance, *P*†			3.3×10^{-8}	5.7×10^{-3}	3.6×10^{-5}
Coefficient of coincidence†			0.29	0.10	0.30

*141 BPs were observed in 1,359 genomic subsections in 10 species. N, *Drosophila novamexicana*; T, *Drosophila texana*; A, *Drosophila americana*; Mo, *Drosophila montana*; La, *Drosophila lacicola*; Ezo, *Drosophila ezoana*; Lu, *Drosophila lummei*; Li, *Drosophila littoralis*; Ka, *Drosophila kanekoi*.
†Obtained by following the statistical procedure described by ref. 18.

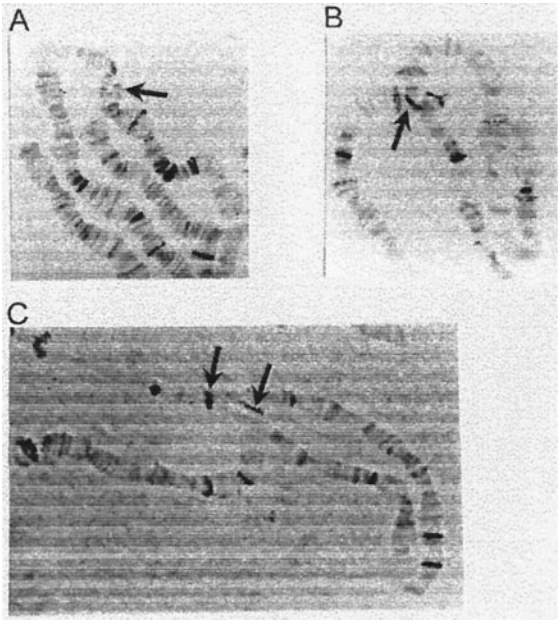

Fig. 2. Examples of chromosomal rearrangements isolated from the progeny of dysgenic crosses. (*A*) Deletion of the distal end of chromosome 5. The arrow indicates the deletion BP. The *Penelope* element present in the 5Ci subsection of strain 160 was probably lost in the process of deletion formation. (*B*) Inversion in chromosome 2. An arrow indicates the inversion BP located in band 2Ij, in which a *Penelope* element is also inserted. (*C*) Inversion in chromosome 3. *Penelope* elements located in both inversion BPs (3Hk and 3Wf) are indicated by arrows.

rearrangements with the locations of inversion BPs previously observed in other species of the *virilis* group (8, 11). A total of 12 coincidences between the subsections of new and old BPs were observed (Table 4, column 6), compared with an expected number of 3.29. The coincidence coefficients were highly significant in every case (Table 4).

Structure of *Penelope* Insertion Sites in *D. virilis* and *D. lummei*. The frequent association between the insertion sites of *Penelope* and *Ulysses* and between these sites and inversion BPs described in species of the *virilis* group, including *D. virilis*, suggests possible hot spots for TE integration. To investigate how precisely the insertion sites are related, a selected homologous chromosomal region of *D. virilis* and *D. lummei* was cloned from each species. This region included an intron of the *eyeless* gene, a cluster of *Pdv* elements (22), and a *Penelope* element insertion. *In situ* hybridization, by using the flanking regions of both genomic clones as probes, enabled us to localize the region to the 49F subsection of chromosome *4*.

Fig. 3 illustrates the structure of this homologous region isolated from genomic libraries of *D. virilis* strain 160 and *D. lummei* strain 203. Both sequences contain *Penelope* elements as well as portions of the same genomic region. However, the two *Penelope* elements are inserted in opposite orientation with respect to the flanking sequence and, in the case of *D. lummei*, only discontinuous fragments of a *Penelope* element are present. Although the two *Penelope* elements apparently inserted in the same general chromosomal region in the two species, they did not do so at identical sites at the nucleotide level.

Alignment of the *D. lummei Penelope* element with a consensus copy of *D. virilis* (p6) indicated many base substitutions, deletions, and insertions in the *D. lummei* sequence (data not shown). The overall nucleotide similarity between the two *Penelope* elements was 82%, ignoring gaps. The *D. lummei* element appears to be a highly diverged defective one that is unable to transpose.

Discussion

It is well known that most mobile elements exhibit some level of insertion site preference (23). However, the degree of target site specificity can vary widely, depending on a number of factors. In this paper, we have shown highly significant deviations from randomness of the distributions of *Penelope* and *Ulysses* insertion sites in the *D. virilis* genome. An excess of subsections in the zero (unoccupied) class accounts for the deviations observed. Statistical analysis indicates that only about one-fifth of the chromosomal subsections is effectively available for occupation by these elements. Statistically significant coincidence coefficients were also observed between the locations of *Penelope* and *Ulysses* insertion sites in *D. virilis* and BPs of chromosomal rearrangements in other species of the group. It seems likely that a common mechanism underlies these nonrandom associations. Because *Penelope* plays a key role in hybrid dysgenesis in *D. virilis* (15), the activity of a *Penelope*-encoded endonuclease seems a likely candidate for determining this insertional specificity.

Penelope is a member of the non-long-terminal repeat (LTR) retroelements (15). Sequence profile analysis suggests that the *Penelope* polyprotein is an active endonuclease that could function as an integrase. It is related to intron-encoded endonucleases and a bacterial repair endonuclease (M.B.E., unpublished results). This type of non-LTR retroposon transposes by cleav-

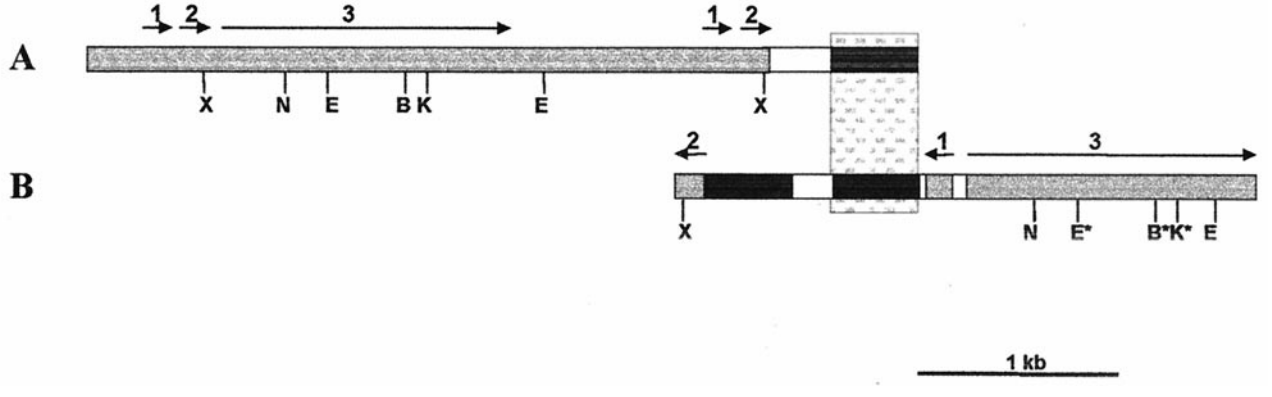

Fig. 3. Schematic representation of structures of *Penelope* elements isolated from genomic libraries. (*A*) A *Penelope*-containing genomic clone of *D. virilis*. (*B*) A *Penelope*-containing genomic clone of *D. lummei*. Gray boxes indicate *Penelope* sequences; black boxes indicate sequences of the *eyeless* gene. The stippled box displays homologous regions of the *eyeless* gene in the two species. White (unshaded) boxes represent genomic sequences of unknown origin. The symbols X, N, E, B, and K represent the restriction sites *Xho*I, *Nde*I, *Eco*RI, *Bam*HI, and *Kpn*I, respectively. Asterisks indicate restriction sites that are damaged in *D. lummei*. Arrows above the boxes indicate the sizes and positions of fragments within a full-length *Penelope* element.

age of the DNA to produce a priming site for reverse transcriptase (23). In some retroposons, such as the *R1* and *R2* families in insects, the high target-site specificity reflects the high specificity of the element-encoded endonuclease (20). Although the limited data suggest that *Penelope* insertion site preference is considerably less specific than that of the *R1* and *R2* retroposons, it appears high enough to produce a strong pattern of nonrandomness. The activity of a *Penelope*-encoded nuclease may also be responsible for breaks in ectopically paired elements that result in rearrangements through the subsequent random rejoining of fragments. This might account for the high coincidence of BPs of newly induced rearrangements with *Penelope* and *Ulysses* insertion sites present in the parental strains.

Genetic and molecular studies indicate that all three systems of hybrid dysgenesis (24) in *D. melanogaster* (P-M, I-R and *hobo*) are accompanied by multiple rearrangements because of recombination between insertion sequences of a single TE family (25–27). However, in contrast to the *D. melanogaster* systems, induced rearrangements in *D. virilis* may be associated with at least two unrelated families of transposons at their BPs. It should be noted that we did not attempt to check whether other TE families that are activated in *D. virilis* hybrid dysgenesis are associated with the BPs of those rearrangements not associated with *Penelope* or *Ulysses*. Such a possibility is currently being investigated (M.B.E., unpublished results).

Results of the present *D. virilis* study show that the chromosomal distributions of *Penelope* and *Ulysses* exhibit significant correlations with the subsections of inversion BPs known for other species of the group. Similar correlations with inversion BPs were reported earlier with respect to the chromosomal distributions of *Penelope* and *Ulysses* in other species of the *virilis* group (18). Because these elements are old components of their genomes, species of the *virilis* group seem likely to share a common pattern of site specificity for *Penelope* and *Ulysses* insertion.

A possible scenario to explain the observed results is as follows. A *Penelope* invasion appears to have taken place at least once early in the history of the *virilis* species group (18). The remnants of that invasion appear to be represented by inactive diverged *Penelope* copies in contemporary species of the *virilis* group (including *D. virilis*), often located in the centric heterochromatin (ref. 18; M.B.E., unpublished results). This earlier *Penelope* invasion is postulated to have activated multiple elements during hybrid dysgenesis and to have induced numerous gross chromosomal rearrangements that led to, or accompanied, speciation in the group. Our recent finding of ancient highly diverged copies of *Penelope* in some strains of *D. virilis,* and in a closely related species, *D. lummei*, strongly supports the sequence of events proposed (M.B.E., unpublished results).

The absence of chromosomal rearrangements in contemporary natural populations of *D. virilis* might be explained by the early divergence of the lineage that gave rise to this species from the rest of the species group. Indeed, it has been postulated that *D. virilis* evolved in southeast Asia, isolated from the remaining species of the group until relatively recently (8). According to our scenario, this divergence would be required to have occurred after the initial *Penelope* invasion but before the massive chromosomal restructuring that occurred in the other species of the group (8). The documented contemporary invasion of *D. virilis* (17) has only recently started to provide the potential for *Penelope*-induced rearrangements in natural populations of this species. It will be interesting to see whether such rearrangements start to appear in natural populations, as they have done in the laboratory.

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