Retrotransposable Elements R1 and R2 in the rDNA Units of *Drosophila mercatorum: abnormal abdomen* Revisited

Harmit S. Malik and Thomas H. Eickbush

Department of Biology, University of Rochester, Rochester, New York 14627

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ABSTRACT

R1 and R2 retrotransposable elements are stable components of the 28S rRNA genes of arthropods. While each retrotransposition event leads to incremental losses of rDNA unit expression, little is known about the selective consequences of these elements on the host genome. Previous reports suggested that in the *abnormal abdomen* (*aa*) phenotype of *Drosophila mercatorum*, high levels of rDNA insertions (R1) in conjunction with the *under-replication* locus (*ur*), enable the utilization of different ecological conditions via a population level shift to younger age. We have sequenced the R1 and R2 elements of *D. mercatorum* and show that the levels of R1- and R2-inserted rDNA units were inaccurately scored in the original studies of *aa*, leading to several misinterpretations. In particular, contrary to earlier reports, *aa* flies differentially underreplicate R1- and R2-inserted rDNA units, like other species of Drosophila. However, *aa* flies do not undergo the lower level of underreplication of their functional rDNA units (general underreplication) that is seen in wild-type strains. The lack of general underreplication is expected to confer a selective advantage and, thus, can be interpreted as an adaptation to overcome high levels of R1 and R2 insertions. These results allow us to reconcile some of the apparently contradictory effects of *aa* and the *bobbed* phenotype found in other species of Drosophila.

THE effect of transposable elements on their host genomes has been a subject of much debate (see Charlesworth and Langley 1989; Charlesworth *et al.* 1994). It is in the transposable element's interest to replicate at a high enough rate to maintain a long-term presence in a particular genome. However, any increase in copy number of transposable elements should be selected against, as it increases the likelihood of a deleterious insertion or ectopic exchange. Some transposable elements have evolved the ability to insert into specific sites within the genome. It should be easier to study and to define the parameters that affect the survival of these site-specific elements.

R1 and R2 elements were originally characterized as type I or II insertions or as intervening sequences in the 28S rRNA genes of *Drosophila melanogaster* (Wellauer and Dawid 1977). It is now clear R1 and R2 are non-long-terminal repeat (non-LTR) retrotransposable elements that have independently managed to carve a niche for themselves by virtue of their ability to site specifically recognize and retrotranspose into sites in the rDNA genes (Xiong and Eickbush 1988; Luan *et al.* 1993). Indeed, R1 and R2 appear to have been present in arthropod lineages since the origin of this phylum, suggesting a highly stable interaction with the host genome (Jakubczak *et al.* 1991; Burke *et al.* 1993, 1998).

Corresponding author: Thomas H. Eickbush, Department of Biology, Hutchison Hall, University of Rochester, Rochester, NY 14627. E-mail: eick@uhura.cc.rochester.edu

The selective consequences and the retrotransposition dynamics of R1 and R2 insertions within a population of organisms remain largely unknown. Insertion of either or both of these elements in a particular rDNA unit switches off expression of that unit (Long and Dawid 1979; Jamrich and Miller 1984). Thus, each transposition event decreases the number of functional rDNA units in a host genome. However, arthropods possess hundreds to thousands of rDNA units, and only a fraction of these units is required for viability. Therefore, the deleterious effect of each R1 and R2 insertion event may be limited. A second factor affecting the survival of these elements is that the rDNA locus itself is subject to high rates of turnover that drive the concerted evolution of the rRNA genes (Dover and Coen 1981). This turnover would presumably eliminate copies of R1 and R2 from the locus. Despite their presumed negative effects on host fitness and the rapid turnover of the rDNA locus, very high levels of R1 and R2 are sometimes found in natural populations of insects, and few, if any, insects are free of their insertions (Jakubczak *et al.* 1991; Lathe *et al.* 1995; Lathe and Eickbush 1997).

In *D. hydei* and *D. melanogaster*, the *bobbed* (*bb*) phenotype is characterized by shortened and abnormally thin scutellar bristles, as well as delayed development to the adult stage (Ritossa 1976; Hawley and Marcus 1989). Previous studies have correlated bristle size and the degree of the *bb* phenotype to either levels of rRNA synthesis (Shermoen and Kiefer 1975) or to the number of uninserted rDNA units (Franz and Kunz 1981). These studies strongly implicate R1 and R2 retrotransposition events (at least indirectly) in causing the *bb* phenotype,

as these events would incrementally reduce uninserted rDNA copy number. The *abnormal abdomen* (*aa*) phenotype was first observed in *D. mercatorum* (Templeton *et al.* 1976). The *aa* phenotype is similar to the *bb* phenotype, although certain life history traits, such as larval cuticle retention in the adult and early fecundity in females, are unique to *aa* (reviewed in Templeton *et al.* 1989).

While *bb* was shown to be dependent only on the number of uninserted rDNA units, the *aa* phenotype had been proposed to be dependent on two closely linked loci: (1) the rDNA locus, in which a third or more of the 28S genes must be inserted by R1 elements (R2 elements went undetected); and (2) the *under-replication* (*ur*) locus, a locus that controls the underreplication of the inserted 28S genes in the polytenization of larval tissues (Templeton *et al.* 1989). In *D. mercatorum*, like other Drosophila species, the entire rDNA locus is underreplicated during the polytenization process (a process called general underreplication; Spear and Gall 1973; Franz and Kunz 1981), with the inserted units being even more underreplicated than the uninserted units (preferential or differential underreplication; Endow and Glover 1979). In *D. mercatorum*, a loss of differential underreplication in a background of high insertion levels was proposed to compound the selective forces acting on the low numbers of functional rDNA units and, thus, result in the *aa* phenotype.

In this report, we reexamine insertions in the rDNA units of *D. mercatorum* and the underreplication of these units in larval tissues. Contrary to previous reports, we show that typical R2 elements do exist in this species and that the rDNA units they occupy were previously scored as uninserted. We further show the presence of a restriction polymorphism in the R1 elements of *D. mercatorum* that was missed in earlier studies. Failure to detect this polymorphism resulted in only a variable fraction of the total R1 elements being scored in the original *aa* studies. These findings seriously affect the model of a linkage disequilibrium between the (lack of) underreplication allele (*ur*) and R1 insertion levels and the type of underreplication occurring in these flies.

MATERIALS AND METHODS

Fly stocks: The following stocks were obtained from the Drosophila stock center (Bowling Green University, Bowling Green, OH): *D. mercatorum* 15082–1521.1, 1521.2, 1521.7, 1521.8, 1521.22, 1521.23; *D. buzzatii* 15081–1291.1; and *D. melanogaster* strain Oregon-R. *D. mercatorum* stock *aa*/*aa* was the kind gift of Dr. Alan Templeton. Except where indicated, *D. mercatorum* strain 1521.1 was used for the sequencing analysis.

PCR amplification, cloning, and sequencing protocols: For the R2 elements, the ribosomal primer 5′-CGTTAATCCATT CATGCGCG-3′ complementary to the 28S sequence starting 31 bp downstream of the R2 insertion site was used in conjunction with the degenerate primer 5′-TCCCARGGNGAYCC NYTNTC-3′ (standard IUPAC nomenclature) coding for the conserved reverse transcriptase amino acid motif QGPDL. After obtaining the sequence of this 1.8-kb segment from the 3′ half of R2, the 5′ half of the element was amplified with a primer to the portion of R2 already sequenced, 5′-TTATCAG CGTTAAGGGTTAG-3′, and the primer 5′-TGCCCAGTGCT CTGAATGTC-3′ complementary to the 28S sequence 60 bp upstream of the R2 insertion site. Thus, complete R2 elements from *D. mercatorum* were obtained in two steps (GenBank accession number AF015685). The sequence of the 3′ end of *D. mercatorum* R1 elements (starting at the conserved reverse transcriptase motif AFADD) has been reported previously (Lathe *et al.* 1995; GenBank accession number U23194). A nondegenerate primer, 5′-GTCAGCATATGCACTGAT-3′, was made to amplify the 5′ half of the R1 elements in conjunction with a primer complementary to the 28S gene upstream of the insertion site, which is described above. The PCR amplification resulted in ~4.0-kb fragments that were cloned and sequenced (GenBank accession number AF015277).

All PCR products were cloned into a modified mp18 vector as described earlier (Burke *et al.* 1995) and completely sequenced using the Universal Sequencing Primer (United States Biochemical, Cleveland, OH) and additional sequencing primers for incremental sequencing. Several clones (at least two in each orientation) were sequenced and collated using the MacVector package of programs (IBI Technologies). Less than 1% nucleotide divergence was detected between any of the R1 or R2 clones. The ORF translations were obtained from the reconstituted DNA sequence. Comparisons of the newly sequenced R2s from *D. mercatorum* and *D. buzzatii*, as well as the *D. mercatorum* R1, to the *D. melanogaster* R2 and R1, respectively, were carried out using the multiple alignment feature of the CLUSTAL W package of programs (Thompson *et al.* 1994).

Because an *Eco*RI restriction site polymorphism was previously noted at the 5′ end of the R1 elements in *D. mercatorum*, but no elements containing the *Eco*RI site were in our PCR clones (see results), we gel purified the size range of 4.2-kb fragments from a genomic DNA *Eco*RI digest. These fragments were then ligated into an *Eco*RI-digested Bluescript plasmid pretreated with calf intestine alkaline phosphatase. After transformation, the colonies were probed with a fragment from an *Eco*RI$^-$ R1 element that had already been obtained. The insert from colonies that hybridized were excised using *Eco*RI/*Bam*HI, cloned into mp18, and sequenced. After sufficient sequence was obtained, a nondegenerate primer was made unique to the *Eco*RI$^+$ R1 element sequence 5′-ATCAGCTG GAGCTGAAGCC-3′, and PCR was carried out on genomic DNA to confirm that the amplified product now contained the *Eco*RI site. Additional clones were thus obtained, and both strands were sequenced after cloning into mp18.

Multiple 5′ junctions from R1 elements were obtained by PCR using primers 120 bp into the R1 element of either the *Eco*RI$^-$ or *Eco*RI$^+$ class in conjunction with an upstream ribosomal primer (described earlier). For doubly inserted (R1 + R2) rDNA units, PCR was carried out using the above *Eco*RI$^-$ R1 5′ primer and a primer 80 bp into to the 3′ untranslated region of the preceding R2 element.

Isolation of tissue-specific DNA: Salivary glands, fat bodies, brains, epidermis, and gut were isolated from the same larva on ice in phosphate-buffered saline (PBS) solution (Ashburner 1989). Tissues from multiple larvae were pooled without sexing, with the assumption that using large numbers of larvae would prevent sex-related biases in different extractions. Genomic DNA was also isolated from individual adult flies (both male and female) as well as pooled adult heads. Genomic DNA was isolated as described earlier (Eickbush and Eickbush 1995).

Genomic blot protocols: Genomic DNA was digested with the appropriate restriction enzyme, electrophoresed on agarose gels (0.8–1.2%), blotted onto nitrocellulose, and probed.

Probes for the data presented in this report were as follows: (1) Figures 6–8, a 280-bp fragment from the 28S gene of *D. melanogaster* immediately downstream of the R1 insertion site (Jakubczak *et al.* 1991); (2) Figure 5, a 530-bp fragment of the 28S gene upstream of the R2 insertion site (generated using primers specific to the 28S gene, 5′-CCAATATCCG CAGCTGG-3′, 500 bp upstream of the R2 insertion site, and 5′-CGTTAATCCATTCATGCGCG-3′, 31 bp downstream of the R2 insertion site); and (3) Figure 8, a 680-bp fragment generated from the *alcohol dehydrogenase* (*Adh*) gene using the degenerate primers 5′-GGCATTGGIY TSGACACCAG-3′ (I represents inosine) and 5′-ACATYCARCCAIGARTTGAAY TTGT-3′. These primers are suitable for amplifying *Adh* fragments from all Drosophila species tested (G. S. Spicer and J. Jaenike, personal communication). A series of R2-specific internal probes, R1-specific internal probes, and 18S rRNA gene probes were also generated and used on genomic blots to confirm restriction fragment maps of the various *D. mercatorum* rDNA units.

Quantitation of genomic blots: After standard hybridization and washing conditions (Jakubczak *et al.* 1992), blots were exposed in a PhosphorImager cassette for varying amounts of time and quantitated in a Molecular Dynamics storm analyzer. In the underreplication experiments (Figures 7 and 8), the same blot was sequentially hybridized with the *Adh* probe and the 280-bp downstream 28S rDNA probe to internally control for amounts of genomic DNA loaded per lane. The levels of different inserted and uninserted rDNA genes are thus measured relative to the *Adh* gene, which serves as an indicator of a characteristic single-copy gene.

RESULTS

Characterization of the R1 and R2 elements of *D. mercatorum*: R2 elements were not detected in previous studies of the rDNA units of *D. mercatorum* (DeSalle *et al.* 1986) and its sister species *D. hydei* (Franz and Kunz 1981). Given the prevalence of R2 elements in all arthropods (Burke *et al.* 1993, 1998), we were interested in whether the *repleta* lineage had experienced a loss of R2. The only other documented R2 loss in Drosophila was in the lineage leading to *D. erecta* and *D. orena* (Eickbush and Eickbush 1995). Using the same PCR primers that were used to amplify R2 from other species of Drosophila, we were able to amplify and partially sequence R2s from *D. mercatorum mercatorum* and *D. buzzatii* (Lathe and Eickbush 1997). R2 elements were also identified by Southern blots in these species and in *D. hydei* (data not shown). Thus, R2 elements were found in all three species tested from the *repleta* group, further confirming the widespread stability of this element throughout the genus Drosophila (Lathe and Eickbush 1997).

We next attempted to confirm that the R1 and R2 elements of *D. mercatorum* represented active lineages. Multiple copies of the R2 elements were PCR amplified in two steps, using degenerate primers corresponding to the conserved reverse transcriptase motifs of R2 elements in conjunction with both the upstream and downstream flanking 28S gene primers (see materials and methods). As with the R2 elements in *D. melanogaster* (Jakubczak *et al.* 1990) and *Bombyx mori* (Burke *et al.* 1987), full-length R2 elements in *D. mercatorum* encode an ~1100-aa open reading frame (ORF). This ORF contains a central reverse transcriptase domain flanked by putative nucleic acid-binding cysteine-histidine motifs (Figure 1). The ORF encoded by the *D. mercatorum* R2 elements did not contain shifts in frame or premature termination codons. In addition, a comparison of a short region of the *D. mercatorum* R2 ORF with the corresponding region sequenced from the *D. buzzatii* R2 ORF revealed a 5.4-fold excess of silent-site over replacement-site substitutions, implying purifying selection (Lathe and Eickbush 1997). None of these features suggests that the R2 element lineage in *D. mercatorum* is inactive. However, as in other species of Drosophila, a significant fraction of the individual copies of these elements are inactive by virtue of 5′ truncations that range in length from a few hundred to thousands of base pairs (data not shown). A complete analysis of the conserved features of R2 elements in all arthropods will be discussed elsewhere (W. D. Burke, H. S. Malik, and T. H. Eickbush, unpublished results).

In the case of the *D. mercatorum* R1 elements, a 1.7-kb fragment corresponding to the 3′ end had already been sequenced (Lathe *et al.* 1995). To fully characterize these insertions in *D. mercatorum*, the ~4-kb segment corresponding to the remainder of the R1 element was PCR amplified and multiple copies were sequenced. As shown in Figure 1, sequencing revealed a typical R1 element containing two ORFs out-of-frame with each other and overlapping for a short distance. Similar to the R1 elements previously characterized in *D. melanogaster* and *B. mori* (Jakubczak *et al.* 1990), the first ORF contained three putative cysteine-histidine motifs, and the second ORF contained a putative apurinic/apyrimidic endonuclease domain, a central reverse transcriptase domain, and a 3′ cysteine motif. Comparison of the R1 sequences with those from *D. hydei* and *D. buzzatii* indicated a 5.9-fold excess of silent-site *vs.* replacement-site substitutions (Lathe *et al.* 1995). Thus, the R1 lineage in *D. mercatorum* also appears active.

Sequence analysis of the R1 element from *D. mercatorum* did lead to one troubling result. The R1 elements sequenced did not have an *Eco*RI restriction site near their 5′ end; thus, they could not have given rise to the 4.2-kb fragment previously identified on the basis of Southern blotting, as rDNA units with R1 insertions (DeSalle *et al.* 1986). In this earlier study, however, a 9- to 10-kb *Eco*RI restriction fragment was evident in the Southern analyses but had not been characterized. Because the *Eco*RI fragment generated by an rDNA unit containing the R1 element we sequenced would be 9.4 kb in length, we hypothesized that this unexplained genomic DNA fragment corresponded to the R1 element we had cloned and sequenced. Southern blots probed with internal fragments of the R1 element confirmed that the 4.2- and 9.4-kb fragments both contained R1 insertions.

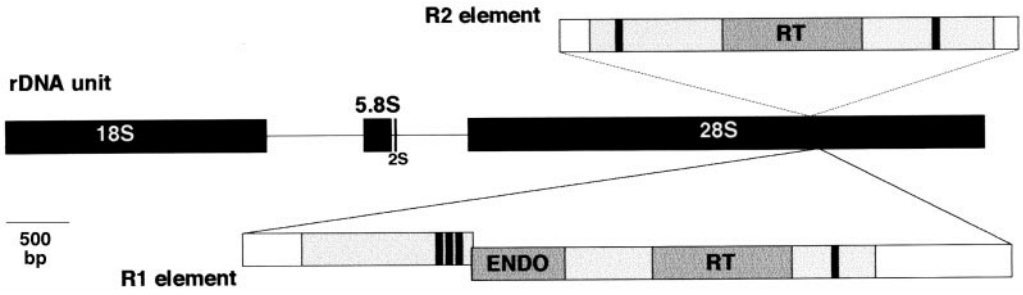

Figure 1.—R1 and R2 elements in the rDNA locus of *D. mercatorum*. The figure represents a single rDNA unit that includes the 18S, 5.8S, 2S, and 28S rRNA genes with internal transcribed spacers. The R1 element encodes two ORFs (shaded boxes) flanked by untranslated regions (open boxes). The first ORF encodes three putative nucleic acid-binding motifs (solid bars), while the second encodes an apurinic/apyrimidic endonuclease (ENDO) and a reverse transcriptase (RT) domain, as well as a 3′ putative nucleic acid-binding motif. The R2 element encodes a single ORF flanked by untranslated regions, and it possesses two putative nucleic acid-binding motifs and a central RT domain.

What, then, was the nature of the R1 element generating the 4.2-kb *Eco*RI fragment detected on Southern blots? Multiple attempts to recover R1 elements with this *Eco*RI site by PCR amplification were unsuccessful. We therefore used a different strategy to clone this R1 variant. An enriched pool of 4.2-kb *Eco*RI fragments was isolated from a genomic DNA digest, ligated into a plasmid, transformed into *Escherichia coli*, and the resulting library was screened for R1 insertions by colony hybridization. Positively hybridizing clones were subcloned into sequencing vectors and shown to contain R1 insertions. These *Eco*RI$^+$ elements were then amplified from genomic DNA using PCR primers specific to this family of elements (see materials and methods).

The sequences of R1 elements with and without the 5′ *Eco*RI site are compared in Figure 2. All differences between the two classes of R1 elements were located within the first 700 bp at the 5′ end of the elements. In this 5′ region, the two classes of R1 are 29% divergent in nucleotide sequence with several length polymorphisms (Figure 2A), while there is <1% divergence within the two classes. These length variants included a duplication of a 55-bp segment in the 5′ untranslated region of the *Eco*RI$^-$ family, as well as the expansion of several regions encoding amino acids that are rich in leucine (L) and proline (P) in the ORF region of the *Eco*RI$^+$ family. One of these coding region expansions was a quadruplication of a 12-nucleotide stretch that encodes for LYPL (Figure 2B). These repeats within the ORF region can potentially form stable hairpins in single-stranded DNA, which may help explain our inability to either amplify or to clone the *Eco*RI$^+$ R1s in competition with the *Eco*RI$^-$ class. All nucleotide changes in the region encoding the ORF were "in frame," suggesting that both families of R1 elements are potentially active. Downstream of this 700-bp region, the two R1 families differed in nucleotide sequence by <1%.

It has previously been argued that R1 elements do not often retrotranspose in the *D. mercatorum* rDNA locus (Hollocher *et al.* 1992). We addressed the issue of retrotransposition (as opposed to recombination) maintaining the level of R1 insertions by examining the 5′ junctions of different copies of the R1 elements within the 28S genes. Retrotransposition events can be followed even though all target sites are equivalent because the mechanism of non-LTR retrotransposition frequently leads to heterogeneous 5′ junctions of the elements with their target sites (reviewed in Luan *et al.* 1993; George *et al.* 1996). Figure 3 summarizes the results from an analysis of R1 5′ junctions in *D. mercatorum*. Panel A shows the sequences of multiple 5′ junctions of the *Eco*RI$^-$ family from two independent geographical strains (1521.1 and 1521.2). While one specific junction predominates, a total of nine and seven distinct junctions were detected in the two strains. A separate analysis of the *Eco*RI$^+$ family in 1521.1 revealed three distinct junctions from five sequenced copies. The 5′ junction variations included the addition and/or deletion of a few bases from the R1 element and nucleotide changes in either the R1 or 28S gene sequence within 10 bp of the junctions. For comparison, in the ~100 bp of flanking 28S gene or R1 sequences, no variation was found between these 52 different R1-containing clones. These results indicate that at least 12 R1 retrotransposition events (combined *Eco*RI$^+$ and *Eco*RI$^-$ classes) must have occurred to explain the current collection of R1 elements in the rDNA units of strain 1521.1.

The 28S gene primer used to amplify the junctions in Figure 3A was located upstream of the R2 insertion site. Thus, all the R1 junctions obtained in that experiment were derived from rDNA units containing only R1 insertions. However, both R1 and R2 elements can insert into the same rDNA unit (Jakubczak *et al.* 1992). To monitor the 5′ junctions of R1 elements that reside in rDNA units also containing an R2 insertion, the experiment shown in Figure 3B was conducted. The upstream PCR primer used in this case was located within the R2 element near its 3′ junction. Of the 17 junctions sequenced, three sequence variants were detected at the R1 5′ junction. These variants were similar to those shown in Figure 3A. Also shown in Figure 3B is the variation found at the 3′ junction of R2 elements. Eleven different R2 3′ junctions were found, all varying in the length of their poly(A) tail. We have previously shown

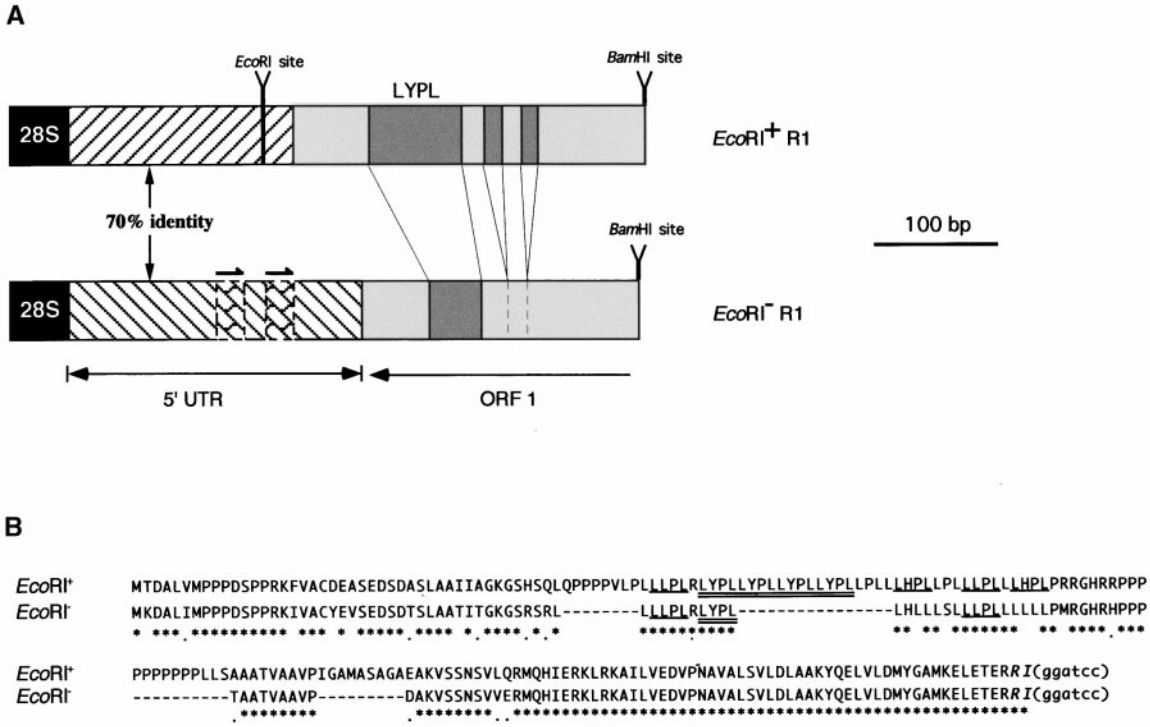

Figure 2.—Comparison of the 5′ ends of the two R1 classes in *D. mercatorum.* (A) Schematic diagram of the sequence differences between the two classes. The 5′ untranslated region of each class is indicated with hatched boxes, while the regions that encode the beginning of the first ORF are indicated by the shaded boxes. The location of the *Eco*RI restriction site polymorphism in the *Eco*RI$^+$ class is shown. A 55-bp duplication within the 5′ untranslated region of the *Eco*RI$^-$ class is indicated by the cross-hatched shading and arrows. Three expansions of the ORF within the *Eco*RI$^+$ class are also shown. Downstream of the common *Bam*HI restriction site, the two classes of elements differ in nucleotide sequence by <1%. (B) Amino acid sequence comparison of the first ORF from the *Eco*RI$^+$ and *Eco*RI$^-$ classes of R1. The LXPL repeats found in the ORF are indicated by single and double underlining. Identical residues are indicated with an asterisk, similar amino acids are indicated by a dot, and gaps inserted to maximize the sequence alignment are indicated with dashes.

that similar poly(A) length variations are generated during the retrotransposition of R2 elements (Luan and Eickbush 1995), and they are characteristic features of the R2 elements of all Drosophila species examined to date (Lathe and Eickbush 1997).

We conclude that the nucleotide variations detected at the 5′ and 3′ ends of R1 and R2 elements of *D. mercatorum* are characteristic footprints of non-LTR retrotransposition. Such variation could be eliminated but not generated by the recombinational mechanisms that lead to the concerted evolution of the rDNA locus. Based on these junction sequence data and the finding that R1 and R2 contained intact ORFs, we argue that these elements have been actively retrotransposing in the *D. mercatorum* lineage and should not be regarded as merely noncoding DNAs (Templeton *et al.* 1989) or as rDNA polymorphisms (Hollocher and Templeton 1994).

Improved blotting method for the detection of R1 and R2 in the rDNA locus of *D. mercatorum*: Based on our sequence analysis of the R1 and R2 elements, a corrected *Eco*RI restriction map of the rDNA locus in *D. mercatorum* can be presented (Figure 4A). The size of each of the restriction fragments was confirmed by a series of genomic DNA blots, using segments of the 28S gene flanking both the 3′ (see Figure 5) and 5′ sides of the insertion sites, internal segments of R1 and R2, and segments specific to the 18S gene as probes. The nontranscribed spacer lengths between two consecutive rDNA repeats are different on the *X* and *Y* chromosomes: 4.4 and 4.1 kb, respectively (see also DeSalle *et al.* 1986). Figure 4B shows a schematic Southern blot of all the hybridizing bands that would be detected if the entire rDNA unit was used as a probe, as in the original *aa* studies. In such a blot, the fragments corresponding to the 3′ end of R2 insertions are 5.3 kb and effectively comigrate with the uninserted rDNA fragments at 5.2 kb. The 3.8-kb fragment corresponding to the 3′ end of R2 would not hybridize intensely with the rDNA probe because of the relatively short length of the hybridizing sequences, and it would readily be lost beneath the major hybridizing bands of 4.4, 4.2, and 4.1 kb. R2-inserted rDNA units were effectively hidden and, therefore, scored as uninserted rDNA units in all previous studies of *aa*.

As a result of the *Eco*RI polymorphism within the R1 elements, the 5′ end of these elements are located on both 4.2- and 9.4-kb fragments. The 3′ ends of both classes of elements generate 1.8-kb fragments, but be-

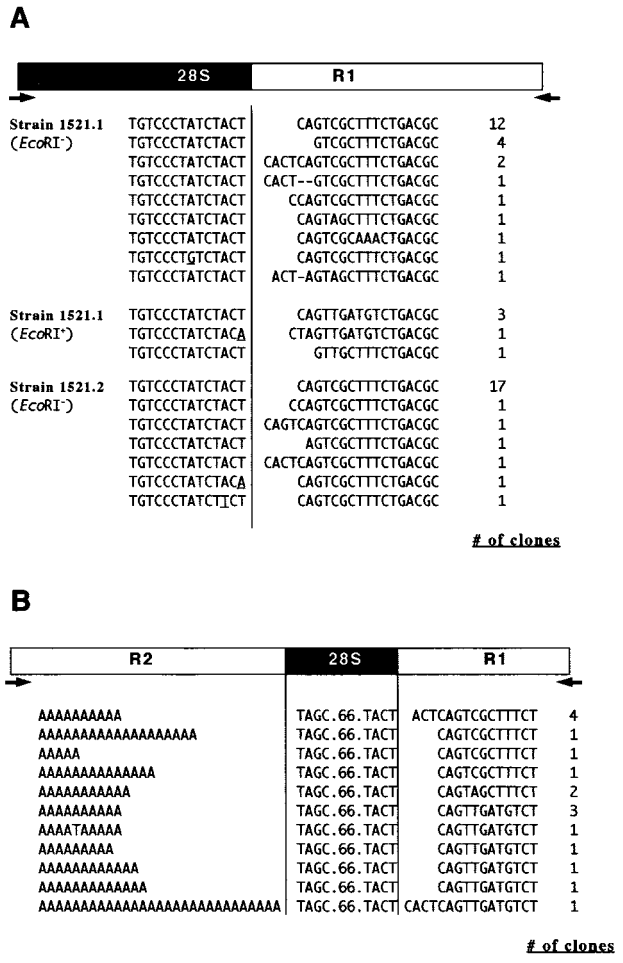

Figure 3.—The 5′ junctions of R1 elements with the 28S rRNA gene. (A) The 5′ junctions of R1 elements singly inserted into rDNA units. The PCR primers used in the amplification were located within the 28S gene, 90 bp upstream of the insertion site and ~120 bp from the 5′ end of the R1 elements. The R1 primers were specific to either the *Eco*RI$^+$ or *Eco*RI$^-$ classes of elements. All sequence variation detected among the individual clones was located at or near the junction of the 28S gene and the R1 element. This variation is a signature of non-LTR retrotransposition events (Luan and Eickbush 1995; George *et al.* 1996). (B) The 5′ junctions of R1 elements inserted into rDNA units also containing an R2 insertion (double inserts). The R2 primer was complementary to a sequence 80 bp from the 3′ end of the element, while the R1 primer was the same *Eco*RI$^-$ primer used for the amplification in A. The 5′ sequence variation of the R1 elements was similar to that in A. Even greater sequence variation was found in the length of the R2 poly(A) tail. The 74 bp of 28S gene located between the R1 and R2 sites exhibited no sequence variation. Only the poly(A) tail at the 3′ end of the R2 elements is shown.

cause these fragments hybridized more weakly to the rDNA probe, the previous authors relied on the 4.2-kb fragment (DeSalle *et al.* 1986). To determine if levels of the two classes of R1 elements varied with respect to each other in *D. mercatorum*, Southern analysis of six different strains of *D. mercatorum* was carried out as illustrated in Figure 5. To simplify the complexity of the blot, we probed the *Eco*RI-digested genomic DNA with a 28S gene sequence 5′ of the R1 and R2 insertion sites. In this blot, uninserted and R2-inserted rDNA units migrate at 5.2–5.3 kb, *Eco*RI$^+$ R1 insertions migrate at 4.2 kb, and *Eco*RI$^-$ R1 insertions migrate at 9.4 kb. The different R1 classes clearly vary independently of each other. Strains 1521.1, 1521.8, and 1521.23 have higher levels of the *Eco*RI$^-$ R1 than the *Eco*RI$^+$ R1 class, while the reverse is true for strain 1521.2. Strains 1521.7 and 1521.22 have similar levels of both classes. Clearly, estimates of total R1 levels based only on estimates of the *Eco*RI$^+$ R1 class would be misleading.

We have previously argued that the most accurate genomic blotting approach to estimate the level of R1 and R2 insertions in the rDNA units of a species uses restriction enzymes that cut near the 3′ end of each element, as well as a hybridization probe that consists of a segment from the 28S gene immediately downstream of the insertion sites (Jakubczak *et al.* 1991). This approach has some major advantages. First, restriction enzymes that give rise to the three bands (R1-inserted, R2-inserted, and uninserted rDNA units) that are well separated and not affected by other components of the rDNA locus can be found readily. Second, using a single short probe that hybridizes equally to both inserted and noninserted units requires no corrections for relative hybridization efficiency. Third, the variation associated with the spacer region of the rDNA unit of most species is eliminated. Fourth, while R1 and R2 elements have homogeneous 3′ ends, many copies of R1 and R2 in the Drosophila species show large 5′ truncations (Jakubczak *et al.* 1992; George *et al.* 1996). Approximately 30% of the R1 and R2 elements of *D. mercatorum* have such truncations (data not shown) and, thus, give rise to a range of weakly hybridizing bands that are not readily scored on genomic blots.

Figure 4C shows a schematic genomic blot of *Eco*RI-digested *D. mercatorum* DNA probed with a short downstream 28S gene probe. The three bands are well separated and easily quantitated. While accurately reflecting the total level of inserted rDNA units, the level of R2 insertions are underestimated by this approach because both R1 and R2 elements can be inserted into the same rDNA unit. If the 28S gene probe is located downstream of the insertions, as in the diagram in Figure 4C, then these double inserts are scored as R1 insertions. If the 28S probe is located upstream of the double insertions, then they are scored as R2 insertions. Unfortunately, as just described, quantitation efforts using upstream 28S probes are less accurate because of the 5′ truncations of some R2 copies.

Underreplication in the ribosomal locus: The genomic blotting method shown in Figure 4C was used to score for levels of R1 and R2 insertions and to monitor the extent of general and differential rDNA underreplication during cycles of DNA endoreplication in larval tissue. We first used this method to directly compare

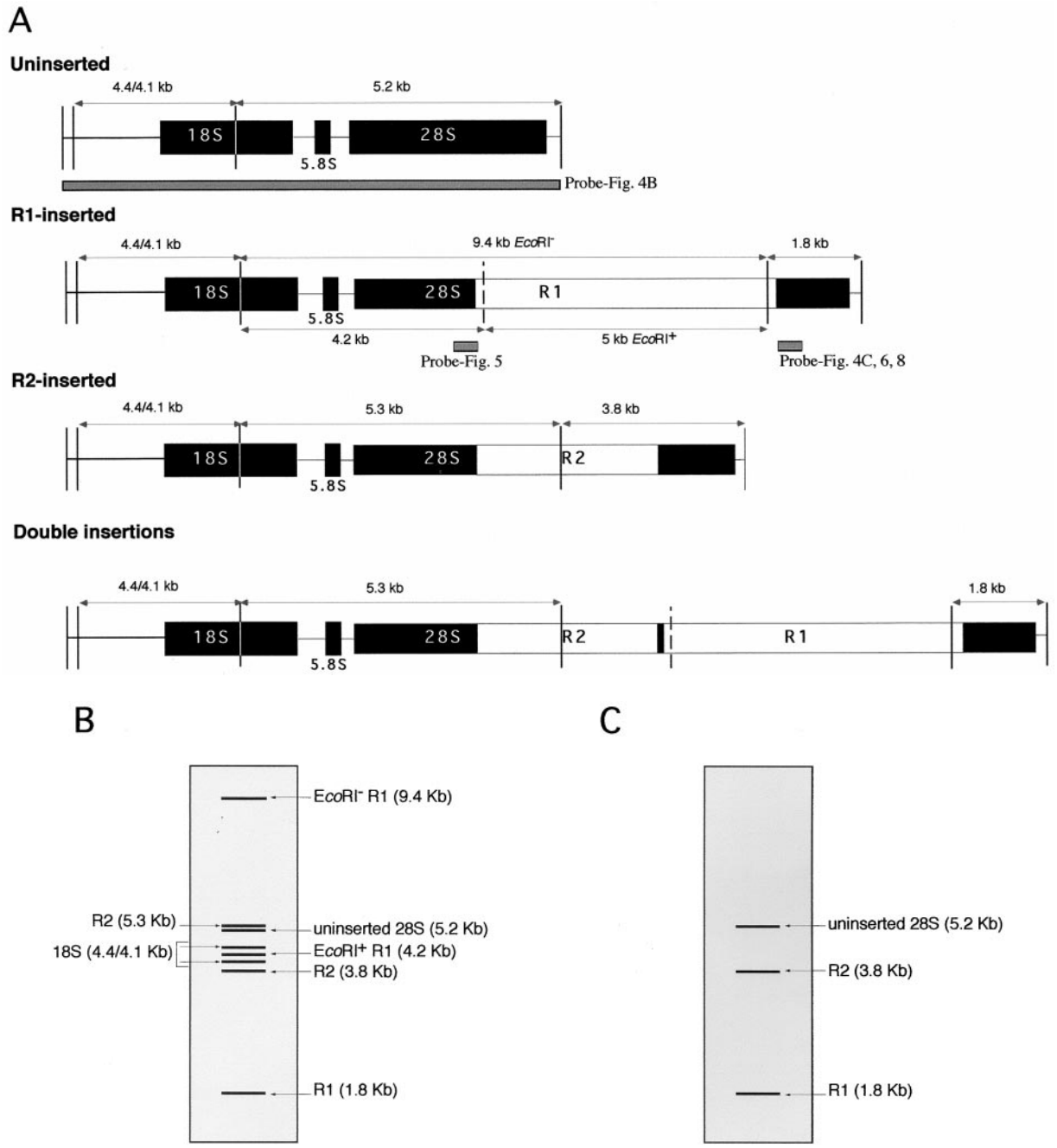

Figure 4.—The rDNA repeat units of *D. mercatorum*. (A) Schematic diagram of the *Eco*RI restriction map of the four types of rDNA units. Vertical lines represent the location of the *Eco*RI sites. The dotted vertical bar at the 5′ end of the R1 element indicates that only one class of R1 elements contains this site. The sizes of the *Eco*RI fragments generated by each unit are shown above the diagrams. The location of the hybridization probes used for the Southerns shown in this report are indicated below the *Eco*RI maps. (B) A schematic genomic blot of *D. mercatorum* genomic DNA digested with *Eco*RI and probed with the entire rDNA unit. The two R2 fragments either comigrate with the uninserted band at 5.2–5.3 kb or are immediately below the 18S/*Eco*RI$^+$ R1 bands. In the case of the two classes of R1 elements, the absence of an *Eco*RI site leads to a 9.4-kb restriction fragment in addition to the 4.2-kb fragment reported in earlier studies (DeSalle *et al.* 1986). (C) A schematic genomic blot of *D. mercatorum* genomic DNA digested with *Eco*RI and probed with a 280-bp 28S gene probe downstream of the R1 and R2 sites. The R1-inserted, R2-inserted, and uninserted rDNA units are well separated. The intensity of the R2 band is an underestimate of the total number of R2 insertions within the locus because many of the R2 insertions occur in rDNA units that also contain an R1 insertion and would be scored as simply R1 inserted.

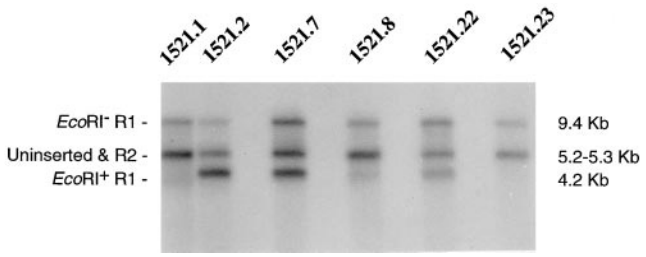

Figure 5.—Southern blot of six different strains of *D. mercatorum*. Genomic DNA from each strain was digested with *Eco*RI and probed with a 530-bp fragment of the 28S gene upstream of the R1 and R2 insertion sites (see Figure 4A). Hybridizing bands represent the comigrating, uninserted, and R2-inserted rDNA units, as well as the two classes of R1 ($EcoRI^+$ and $EcoRI^-$). The proportions of the two classes of R1 elements vary independently of each other in the six strains.

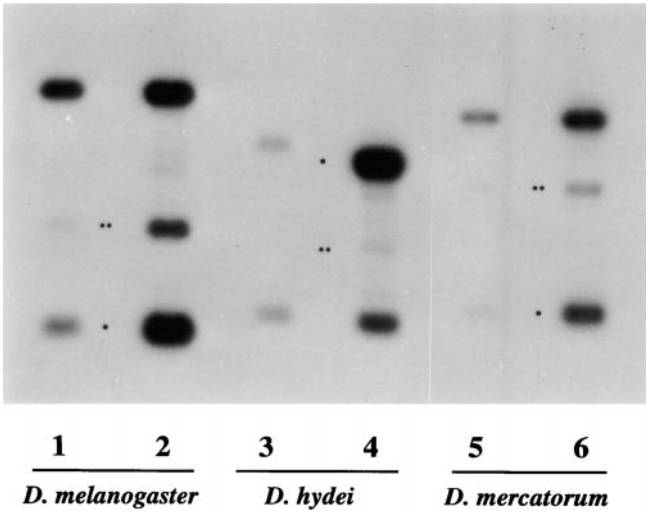

Figure 6.—Underreplication of the rDNA units in three Drosophila species. Shown are Southern blots of pooled polytene salivary glands DNA (lanes 1, 3, and 5) and pooled diploid adult heads DNA (lanes 2, 4, and 6) from *D. melanogaster, D. hydei, and D. mercatorum. D. melanogaster* DNA was digested with a combination of *Cla*I, *Bam*HI, and *Eco*RI (see Jakubczak *et al.* 1992 for restriction maps of the rDNA units of this species), *D. hydei* DNA was digested with *Cla*I and *Hin*cII, and *D. mercatorum* DNA was digested with *Eco*RI. The DNA was hybridized with a 28S gene probe located downstream of the R1 and R2 insertion sites (see Figure 4A). Hybridizing fragments representing R1- and R2-inserted rDNA units are represented by single and double dots, respectively. The *D. hydei* salivary gland DNA contained salt, which resulted in the slower migration of the bands compared to head DNA. While both inserted and uninserted rDNA units are underrepresented in the polyploid tissues, the inserted rDNA units are more severely underrepresented. Thus, general and differential underreplication is qualitatively similar in all three Drosophila species.

the underreplication processes in the three species that have previously been studied: *D. melanogaster* (Endow and Glover 1979), *D. hydei* (Franz and Kunz 1981), and *D. mercatorum* (Templeton *et al.* 1989). Figure 6 shows genomic DNAs from polytene salivary gland tissues (lanes 1, 3, and 5) and the diploid tissues of adult heads (lanes 2, 4, and 6) digested with an appropriate restriction enzyme, blotted, and probed with a downstream 28S gene fragment (see Figure 4A). Equal amounts of DNA were loaded in each lane to enable a direct estimate of the level of underreplication in the polytene tissue. The blots indicate that in each species the total level of rDNA units hybridizing in the polytene tissue is less than in the diploid tissue. The reduction in the level of the uninserted rDNA units appears to be approximately 2- to 4-fold, while the reduction in the level of those units containing insertions [bands indicated by single (R1) and double (R2) dots] is more extensive and can be estimated at close to 10-fold. Thus, while the total number of rDNA units has undergone a decrease in polyploid cells, a significantly smaller fraction of the rDNA units that remain are inserted with R1 and R2.

We next investigated the effect of the ploidy level of a tissue on the degree of rDNA underreplication. We also wanted to quantify the absolute level of underreplication in these studies because it is the final number of functional rDNA units (and not their proportion to inserted units) that is likely to affect fitness. To quantify the underreplication of the rDNA units, the genomic blots were probed with both an *Adh* gene sequence (as a representative single-copy gene) and the 28S gene sequence (for both inserted and uninserted rDNA units). We chose larval brains and adult heads as representative of diploid (2N) tissues, larval epidermis and midgut as representative of intermediate levels of polyteny-ploidy (64N), and larval fat bodies (256N) and salivary glands (1024-2048N) as tissues with maximum levels of polyteny (Ashburner 1989).

Figure 7 summarizes the quantitation of the results of the genomic blotting experiment with wild-type *D. mercatorum* polypoid tissues. The *X*-axis of this graph reflects the number of replication cycles required to reach a certain level of ploidy (which equals $\log_2$ [ploidy]). The *Y*-axis of the graph reflects the levels of each type of rDNA unit relative to *Adh*, scaled with the diploid complement arbitrarily set as 1. As shown, the degree of ploidy has a direct bearing on the degree to which a particular tissue is underreplicated, suggesting that the underreplication process is gradual. Every replication cycle results in a further decrease in rDNA units per genomic equivalent relative to diploid tissue for both the inserted and uninserted units; thus, the degree of underreplication per round of replication appears to be fairly constant in different polyploid tissues. We have obtained similar results in another strain of *D. mercatorum* and in two different strains of *D. melanogaster* (data not shown).

In Figure 8, we show the results of a similar experiment using a strain of *aa* flies kindly provided by Dr. A. Templeton. While this is the only strain of *aa* that was available to us, it should be noted that this strain was used in the original studies of this phenotype. Figure

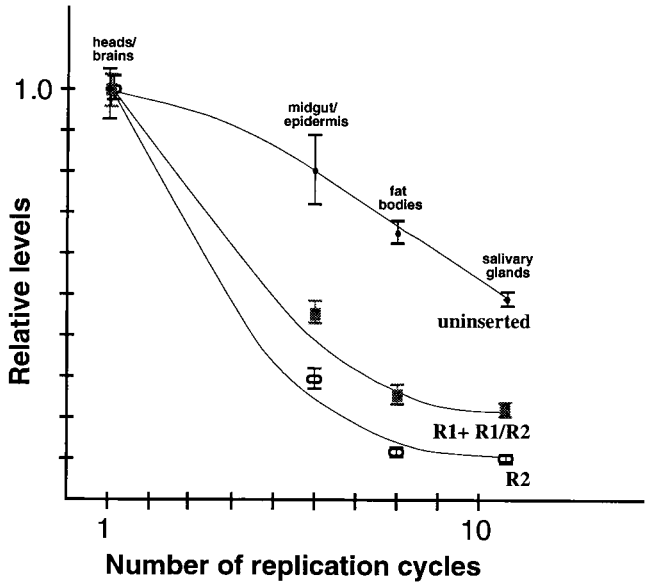

Figure 7.—Underreplication of the rDNA units in the polyploid tissues of wild-type *D. mercatorum*. DNA was isolated from a series of larval and adult tissues that represent varied levels of ploidy, and it was probed consecutively with both the same 28S gene probe DNA used in Figure 6 and an *Adh* probe (see materials and methods). Tissues included are as follows: diploid (adult heads, larval brains), 64-ploid (larval epidermis, midgut), 256-ploid (larval fat bodies), and 1028- to 2056-ploid (larval salivary glands). Measurements of each type of rDNA unit are scaled relative to *Adh* levels measured for the particular genomic DNA. To illustrate levels of general and differential underreplication, all three types of units are represented as arbitrarily scaled, with the diploid complement representing 1. Both general and differential underreplication are clearly dependent on the level of ploidy.

8A shows the actual Southern hybridization of the *aa* strain DNA with the blot of the *Adh* gene probe shown below that of the rDNA probe. These data are graphed in Figure 8B in a manner similar to that in Figure 7. Before comparing the underreplication data, two differences can be seen when comparing the rDNA blot of diploid DNA obtained from *aa* flies (Figure 8, lane 1) with that of the wild-type flies (Figure 6, lane 6). First, the level of R1 and R2 insertion is considerably higher in the *aa* strain. Only 14% of the rDNA units are uninserted, compared to ≥30% uninserted in all the wild-type *D. mercatorum* strains we have tested. Second, a fraction of the R1 and R2 insertions do not comigrate with the major R1- and R2-inserted bands (fainter bands above and below the uninserted and R2 bands). These additional restriction polymorphisms complicate determinations of the total fraction of the units inserted with R1 and R2, but they do not affect our ability to quantitate the level of underreplication in the bands we can score.

Comparison of the underreplication of rDNA units in *aa* and wild-type flies reveals one major difference. The uninserted rDNA units of the *aa* flies do not undergo general underreplication. On the other hand,

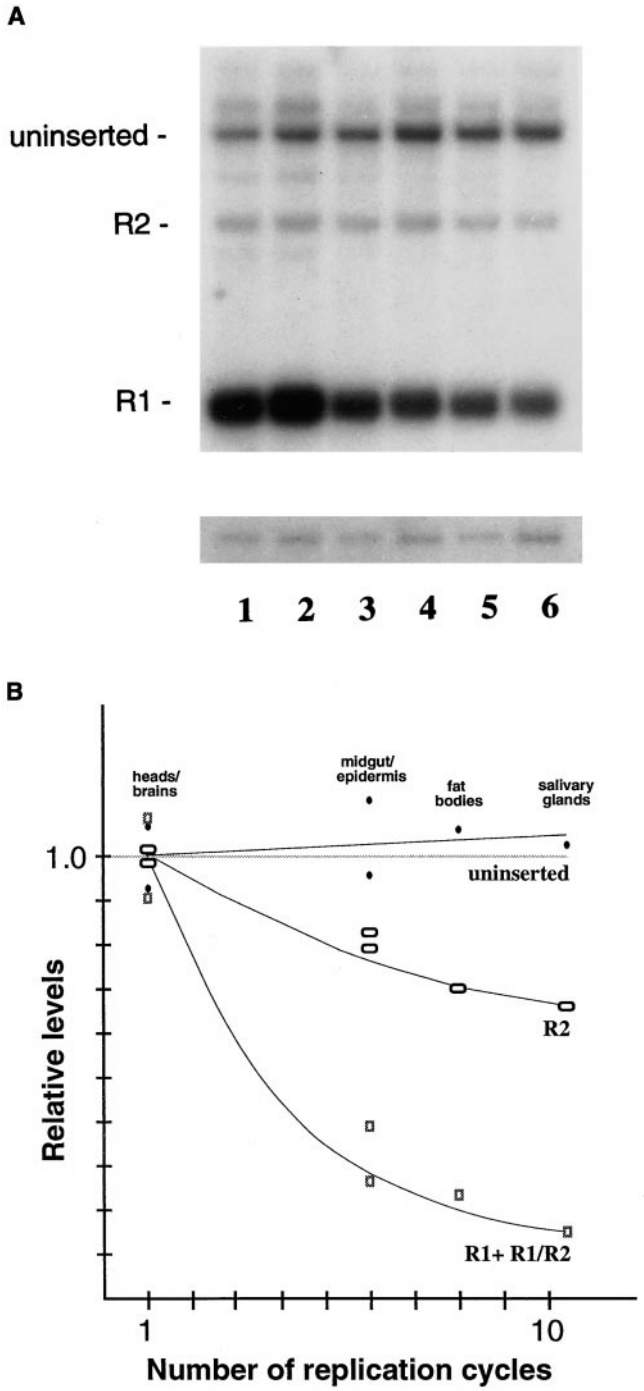

Figure 8.—Underreplication of the rDNA units in the polyploid tissues of *aa* flies of *D. mercatorum*. (A) Southern analysis of tissues of various ploidy levels from *aa* larvae and adults. Lane 1, adult head; lane 2, larval brain; lane 3, larval epidermis; lane 4, larval midgut; lane 5, larval fat bodies; lane 6, larval salivary glands. Southern blots were probed with both the downstream 28S gene probe (see Figure 4A, top panel) and an *Adh* probe (Figure 4A, bottom panel). (B) Quantitation of general and differential underreplication in *aa* flies was conducted as described in Figure 7. The general underreplication is completely ameliorated in larval tissues at every ploidy level, while differential underreplication is still observed.

differential underreplication of the inserted rDNA units does occur in *aa* flies. The level of this differential underreplication in the *aa* flies is approximately similar to that of the wild-type flies if one corrects for the twofold general underreplication in the wild-type flies. The fact that differential underreplication of inserted rDNA units occurs in *aa* flies was unexpected, as the lack of differential underreplication was proposed to be a key determinant of *aa* (Templeton *et al.* 1989). The difference between the results in Figure 8 and those published previously can be explained in part by the R2 insertions in the rDNA locus. We estimate the level of R2 insertion in the *aa* line to be from 15 to 25%. A more accurate estimate is difficult, as most of the R2 elements reside in rDNA units that also contain R1 elements (see previous section). Because the restriction fragments derived from the R2-inserted rDNA units comigrated with those from the uninserted rDNA units in the previous reports, the previous authors were misled into thinking that the ratio of inserted and uninserted units remained constant in polyploid tissues. This error went undetected because only the relative levels between the hybridizing bands were being scored, not the absolute level of rDNA units within the genome. As discussed below, the lack of general underreplication in *aa* organisms compared to wild type suggests that the change in underreplication that occurs in *aa* flies is similar to that of rDNA compensation seen in *D. melanogaster* and *D. hydei* (Spear and Gall 1973; Franz and Kunz 1981).

DISCUSSION

The R1 and R2 retrotransposable elements are highly stable, long-term components of arthropod genomes (Burke *et al.* 1998). The only known case of elimination of either of these elements from a genome is found in Drosophila, where a survey of 59 species from 23 species groups found R2 elements absent in only one lineage of the melanogaster subgroup that contains *D. erecta* and *D. orena* (Eickbush and Eickbush 1995; Lathe *et al.* 1995; Lathe and Eickbush 1997). Clearly, there has been ample time for arthropod genomes to adapt to these elements. However, the data available to date suggests that these elements are, for the most part, deleterious. Each retrotransposition event leads to an incremental decrease in the number of functional rDNA units (Long and Dawid 1979; Jamrich and Miller 1984), and high levels of insertions have been linked to the *bb* phenotype (Franz and Kunz 1981).

Templeton and co-workers (Templeton *et al.* 1985, 1989; DeSalle and Templeton 1986; DeSalle *et al.* 1986) proposed that the *aa* phenotype in *D. mercatorum* is caused by a combination of two factors: high levels of inserted rDNA units and a gene controlling the levels of differential underreplication in polytene tissues. The authors contended that *aa* flies lacked the ability to differentially underreplicate inserted units relative to uninserted units, whereas wild-type flies had this ability. This loss of function caused an "effective overreplication" of inserted rDNA units relative to the uninserted units. When levels of insertions and differential underreplication in a natural population in Hawaii were scored, a statistical cosegregation of the two determinates required for the *aa* phenotype was found. The authors contended that because neutral conditions could not explain such an association, it must reflect a selective advantage, most likely caused by a shift in the population towards a younger age structure, which, in Hawaii, was hypothesized to be adaptive at times of low rainfall (Hollocher *et al.* 1992; Templeton *et al.* 1993; Hollocher and Templeton 1994). Thus, in effect, these studies suggested an adaptive explanation for higher levels of insertions that inactivate the rDNA units of an organism.

Here we have shown that levels of inserted rDNA units were incorrectly scored in previous studies. R2 elements are abundant in *D. mercatorum*, but were scored as uninserted units. Furthermore, a variable fraction of the R1 insertions containing a restriction polymorphism (the *Eco*RI$^-$ subfamily) were ignored. Because it is impossible to extrapolate total insertion levels based on the fraction of (*Eco*RI$^+$) R1s that were scored, our results call into doubt any finding of linkage disequilibrium between the presence of the recessive allele at the *ur* locus and high levels of R1 insertions in the rDNA locus, *i.e.*, the supergene hypothesis (Hollocher *et al.* 1992). We also showed that because the previous studies could not distinguish between R2-inserted and uninserted rDNA units, lack of general underreplication was mistaken for lack of differential underreplication. The lack of differential underreplication of the inserted rDNA units can be proposed to be deleterious in larval tissues; *e.g.*, an excess number of inserted rDNA units could soak up transcription factors. However, a lack of general underreplication of all rDNA units in *aa* flies implies a greater number of active rDNA units per polyploid cell, which would presumably be advantageous.

Based on our findings, we offer an alternate explanation of the *aa* phenotype. *aa* flies have very high levels of rDNA insertions (the strain we tested had the highest level of insertions we have found in any insect tested to date). Flies containing such high levels of insertions are at a disadvantage. However, this disadvantage is largely ameliorated by the lack of general underreplication in polytenization, which results in a larger number of active rDNA units per cell. In spite of this increase, if the starting complement of rDNA units is not sufficient, some tissues may still have fewer than optimal numbers of uninserted rDNA genes. For example, *aa* is clearly a defect of the larval fat bodies in which insufficient amounts of the juvenile hormone esterase (required to break down levels of juvenile hormone) leads to a persistence of the juvenilized phenotype in the adult fly (Templeton and Rankin 1978). Our alternative

model for *aa* offers a very different view of the impact of R1 and R2 elements in the genome of *D. mercatorum*. Instead of using high levels of insertion to exploit an adaptive niche, as proposed in the earlier studies, *aa* flies have invoked an adaptive response to counter higher levels of R1 and R2 insertions. In other words, the failure of general underreplication at extremely high levels of insertion reflects the flies' attempt to overcome the otherwise deleterious consequences of these elements.

Second locus (*ur*) or threshold effect? The existence of a second locus, *ur*, was proposed previously on the basis of several genetic experiments in *D. mercatorum* (Templeton *et al.* 1985). First, Templeton and co-workers crossed a low-insertion strain K28-O-Im and an *aa* strain to show that loss of differential underreplication could be scored, even in a line that bore low levels of rDNA insertions. Second, to map the additional determinant(s) of *aa*, two strains, S and K, were crossed to the *aa* tester stocks. These crosses indicated that while the *X* chromosome of the K strain supported the manifestation of the *aa* phenotype, the *X* chromosome of the S strain did not. Neither the K nor S strains themselves exhibited the *aa* phenotype. If, however, F_1 females from a K × S cross were allowed to reproduce parthenogenetically, 0.4–0.8% of the parthenogenetic F_2 displayed the *aa* phenotype. The authors concluded from these experiments that two genetic loci were required for *aa*, and these loci were on the order of one map unit apart.

We propose that both of these experiments can be readily interpreted in terms of a threshold effect being responsible for *aa*. Visual inspection of a Southern blot of the K28-O-Im line indicated that although the level of the *Eco*RI$^+$ R1s that were scored is indeed low, the *Eco*RI$^-$ R1s are actually at moderate levels (DeSalle and Templeton 1986; Figure 4), and, thus, the K28-O-Im line is not a low-level insertion line. Second, the authors appear to dismiss the possibility that the parthenogenetic *aa* F_2 progeny of the K *vs.* S crosses could result from recombination within the rDNA locus rather than between the rDNA locus and a second determinant. Recombination within the rDNA loci could likely lead to recombinant progeny with a deficiency of functional rDNA units.

This threshold model is also supported by data showing that the determining factor for the *aa* phenotype in males was on the *Y* chromosome. The previous authors have postulated that the cause of the *aa-Y* chromosomes obtained were probably deletions of the rDNA units from the *Y* chromosome (Templeton *et al.* 1985; Hollocher *et al.* 1992). Thus, consistent with the model that it is the total number of uninserted units that determine the *aa* phenotype, they found that any *aa-Y* chromosome could be rescued by a non-*aa-X* chromosome, while any *aa-X* chromosome could be rescued by a non-*aa-Y* chromosome.

The pleiotropic effects of *aa* and *bb*: Both *bb* and *aa* flies cover a range of phenotype severity, a continuum presumably imposed by the incremental loss or gain of functional rDNA units. The present analysis affords an excellent opportunity to compare and contrast the causes and phenotypes of *bb* and *aa*. *bb* flies are characterized by a number of phenotypic aberrancies, including shortened bristles, abnormal abdominal sclerites, longer emergence times, delayed maturity, higher sterility, and lower longevity (Ritossa 1976). *aa* flies share some of the same phenotypes as *bb*, but also some distinct characteristics. If, as shown in this report, *aa* flies are able to undergo compensation during polytenization while *bb* flies are not (Franz and Kunz 1981), some of these differences can be explained. For example, increased female fecundity in *aa* flies (Templeton *et al.* 1993) may be because the nurse cells of the meroistic ovaries in dipterans are polyploid (Renkawitz-Pohl and Kunz 1975a,b). In the maturation stages of the oocytes, ribosomes are produced in these nurse cells and transported into the developing oocytes. The absence of general underreplication in *aa* flies may mean that nurse cells in these flies are actually more proficient at manufacturing ribosomes than in their wild-type counterparts (that are also facing high levels of rDNA insertions). Thus, rDNA compensation may account for higher levels of fecundity observed in *aa* females. Polyploid cells have not been observed in sperm development, thus the inability to compensate for high levels of rDNA insertions during spermatogenesis would delay sexual maturation in *aa* males.

In a similar manner, the observation that *bb* flies contained shortened bristles on the adult cuticle while *aa* flies do not can also be explained by differences in underreplication. Because the bristle-forming cells are polyploid (Overton 1967), compensation in *aa* flies gives rise to normal bristle development in the patches of the abdomen that are not juvenilized, while the lack of compensation leads to shortened bristles in *bb* flies. Further analysis should reveal whether more of the differences between the *aa* and *bb* phenotypes can be directly traced to the ploidy of the tissues involved.

The retrotransposable elements R1 and R2: We have previously presented data suggesting that R1 and R2 elements have been present as independently transposing entities in the rDNA locus since the origin of the phylum Arthropoda (Burke *et al.* 1998). What explains this remarkable success story? Although the null hypothesis remains that these elements are "selfish DNA" entities, one has to entertain the possibility that these elements provide some benefit to the host. It has been proposed that this role could be to stimulate recombination between rRNA genes, in effect driving concerted evolution by making endonucleolytic cleavages within the rDNA locus (Hawley and Marcus 1989). An alternative proposal is that R1 and R2 make a factor that participates in the normal expression of the rDNA genes. Although these proposals are certainly feasible,

one would expect that the host genome would have found an opportunity to usurp the site-specific endonuclease or regulatory functions in the greater than 500 million-year history of arthropods. In addition, the presence of two stable lineages of independent, transposable elements to carry out the same primary function is not consistent with these proposed roles.

The only other beneficial role that has been proposed for R1 and R2 elements was that the elements controlled the downregulation of rDNA units. This proposal is derived from studies of *aa* in *D. mercatorum*, which suggest that under certain environmental conditions, the delayed development and early fecundity life history tradeoffs caused by high levels of insertions in *aa* flies may have advantages (Templeton *et al.* 1989). We have called this model into question because our data suggest that *aa* flies are attempting to overcome the problem associated with high levels of insertion. However, even in the absence of our new data, if the changes in life history traits associated with *aa* flies in *D. mercatorum* merely result from a lack of sufficient numbers of functional rDNA units, it would seem that a simpler solution for the host genome would be to have a "smaller" rDNA locus or to downregulate expression rather than sponsor two independent, retrotransposable elements to do this job.

The transposition of R1 and R2 elements in the rDNA locus of arthropods can be translated analytically to a simple mutation-selection (retrotranspositions-rDNA units) balance. If anything, the maintenance of a naturally occurring compensatory response (lack of general underreplication) in *D. mercatorum* could point to high retrotransposition rates that affect these populations. Thus, we have to consider that the ability of R1 and R2 elements to have thrived in the rDNA locus of arthropod genomes for more than 500 million years is a testament to their ability to retrotranspose at frequencies that are high enough to ensure survival.

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