Dynamics of R1 and R2 Elements in the rDNA Locus of *Drosophila simulans*

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ABSTRACT

The mobile elements R1 and R2 insert specifically into the rRNA gene locus (rDNA locus) of arthropods, a locus known to undergo concerted evolution, the recombinational processes that preserve the sequence homogeneity of all repeats. To monitor how rapidly individual R1 and R2 insertions are turned over in the rDNA locus by these processes, we have taken advantage of the many 5′ truncation variants that are generated during the target-primed reverse transcription mechanism used by these non-LTR retrotransposons for their integration. A simple PCR assay was designed to reveal the pattern of the 5′ variants present in the rDNA loci of individual X chromosomes in a population of *Drosophila simulans*. Each rDNA locus in this population was found to have a large, unique collection of 5′ variants. Each variant was present at low copy number, usually one copy per chromosome, and was seldom distributed to other chromosomes in the population. The failure of these variants to spread to other units in the same rDNA locus suggests a strong recombinational bias against R1 and R2 that results in the individual copies of these elements being rapidly lost from the rDNA locus. This bias suggests a significantly higher frequency of R1 and R2 retrotransposition than we have previously suggested.

RIBOSOMAL RNA genes are encoded by large multiunit arrays located in the nucleolar organizer region of all eukaryotes (Long and Dawid 1980). Each tandem repeat is composed of an 18S, 5.8S, 2S, and 28S rRNA gene organized as a single transcription unit. These "rDNA" loci are subject to concerted evolution, a process that preserves sequence homogeneity within the array, but allows the sequence of the entire array to change over time (Arnheim 1983). Concerted evolution in the rDNA locus is believed to be driven by the recombinational mechanism of unequal crossing over and gene conversion (Szostak and Wu 1980; Coen *et al.* 1982; Dover *et al.* 1982; Dvôrák *et al.* 1987).

R1 and R2 are site-specific non-long-terminal-repeat (LTR) retrotransposons that have been identified in the rDNA loci of every arthropod lineage examined to date (Jakubczak *et al.* 1991; Burke *et al.* 1993, 1998). Both elements insert into specific sequences in the 28S gene with the R1 insertion site located 74 bp downstream of the R2 insertion site (Figure 1). Individual 28S genes can contain either one or both elements (Jakubczak *et al.* 1990, 1992). Insertion of either R1 or R2 inhibits transcription of the 28S gene, effectively inactivating the rDNA unit (Long and Dawid 1979; Kidd and Glover 1981; Jamrich and Miller 1984). R1 and R2 elements have been vertically inherited for long periods of evolution (Eickbush *et al.* 1995; Lathe *et al.* 1995; Lathe and Eickbush 1997; Gentile *et al.* 2001), possibly since the origin of arthropods (Burke *et al.* 1998, 1999).

R1 and R2 elements should be subject to the same forces of concerted evolution as the rDNA genes themselves. While a number of studies have monitored the distribution of sequence variants within the Drosophila rDNA loci in attempts to understand the process of concerted evolution, these studies have focused on the spacer regions of the units and largely ignored the R1 and R2 elements (Linares *et al.* 1994; Schlötterer and Tautz 1994; Bowen and Dover 1995; Polanco *et al.* 1998, 2000). Evidence that these elements may also be undergoing concerted evolution includes the very high level of sequence identity that has been determined for different copies of each element within a species (Eickbush *et al.* 1995; Lathe *et al.* 1995; Lathe and Eickbush 1997). A dynamic turnover of these elements in each species is also suggested by the wide range in the fraction of the rDNA units occupied by the elements in different individuals of the same species. Different geographical isolates, and even single populations of *Drosophila melanogaster*, have been shown to have 5- to 15-fold differences in R1 and R2 insertion levels (Lyckegaard and Clark 1991; Jakubczak *et al.* 1992).

To accurately monitor the turnover of R1 and R2 within the rDNA locus requires the ability to follow individual copies of each element. Fortunately, such individual copies can be scored because the target primed reverse transcription (TPRT) mechanism used by these elements for integration frequently generates variable length deletions at their 5′ end (Luan *et al.* 1993). These 5′ truncations are generated because integration proceeds normally even when the reverse tran-

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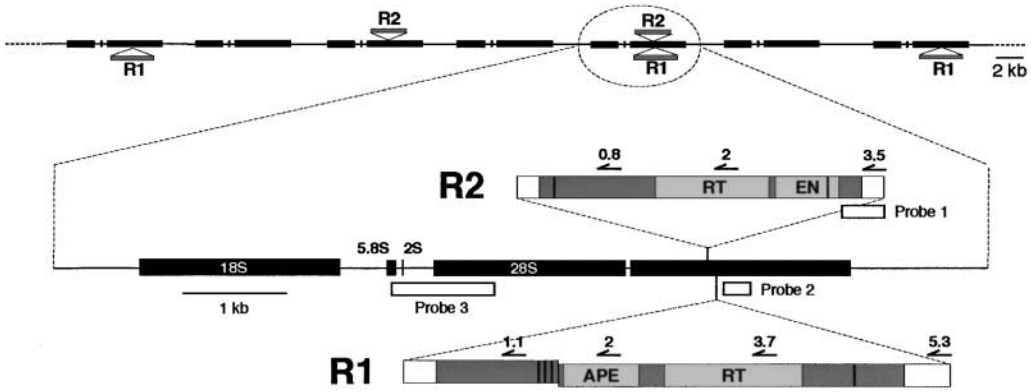

FIGURE 1.—Organization of R1 and R2 elements in the rDNA loci of Drosophila species. The rDNA loci are composed of hundreds of tandemly repeated rDNA units. Each rDNA unit contains an 18S, 5.8S, 2S, and 28S rRNA gene (solid boxes) separated by internal transcribed spacers. R1 and R2 elements insert into specific sites in a fraction of the 28S genes. Individual 28S genes can contain either or both insertions. Shown at the bottom of the figure is an expanded view of an individual rDNA unit containing both an R1 and R2 element. R1 elements encode two open reading frames (ORF) and R2 elements a single ORF (shaded boxes) flanked by untranslated regions (open boxes). Within these ORFs the location of apurinic/apyrimidic endonuclease (APE), reverse transcriptase (RT), and C-terminal endonuclease (EN) domains is shown in lighter shading. Solid vertical lines within the ORFs correspond to cysteine motifs that are believed to be important for nucleic acid binding. The location of probes used for the genomic blots in this article are shown and described in MATERIALS AND METHODS. The location of PCR primers used to monitor the 5′ truncations of the R1 and R2 elements are denoted by half arrows with numbers corresponding to their location in kilobases from the 5′ end of the element.

scriptase fails to reach the 5′ end of the RNA template (see discussion in EICKBUSH 2001). All non-LTR retrotransposons generate a wide range of 5′ truncations. For example, >90% of the L1 elements in humans contain 5′ truncations involving 100s to 1000s of base pairs (KAZAZIAN and MORAN 1998). These truncations are readily duplicated in *in vivo* assays of retrotransposition (MORAN *et al.* 1996; EICKBUSH *et al.* 2000). Once a copy of R1 or R2 is integrated into the rDNA locus, recombination does not appear to generate additional deletions, because no R1 or R2 element has yet been identified that contains deletions of their 3′ end (EICKBUSH and EICKBUSH 1995; LATHE *et al.* 1995; LATHE and EICKBUSH 1997; GENTILE *et al.* 2001). Thus, 5′ truncation variants serve as convenient footprints of retrotransposition events.

In this study we have examined the turnover of individual R1 and R2 elements by monitoring the 5′ sequence variants in a population of *D. simulans*. Two properties of their rDNA units suggested this turnover would be easier to monitor in *D. simulans* than in *D. melanogaster*. First, the rDNA units in *D. simulans* exist only on the X chromosome (LOHE and ROBERTS 1990; MECHEVA and SEMIONOV 1992), while they exist on both the X and Y chromosomes in *D. melanogaster* (LONG and DAWID 1980). Second, *D. simulans* contains significantly lower levels of R1 insertions than *D. melanogaster* (EICKBUSH and EICKBUSH 1995). Concerted evolution would be expected to expand the number of copies of some individual 5′ sequence variants both within a rDNA locus and between rDNA loci. Our study showed, however, that 5′ variants seldom spread to other units within or between loci, suggesting that individual variants of R1 and R2 elements are rapidly eliminated by the recombinational processes of concerted evolution.

MATERIALS AND METHODS

Fly stocks and DNA isolation: Thirty-three isofemale lines of *D. simulans* collected in 1995 at the Noble Apple Orchard in Paradise, California, were a kind gift of M. Turelli. Initially, 5–10 individual flies from several of these lines were monitored for 5′ truncations using the PCR protocol described below. Different flies from the same line, whether male or female, had identical or nearly identical truncation profiles, confirming that few, if any, rDNA units were present on the Y chromosome (LOHE and ROBERTS 1990; MECHEVA and SEMIONOV 1992) and that each line contained a single fixed X chromosomal rDNA locus. Therefore, for the experiments described in this article, DNA was isolated (EICKBUSH and EICKBUSH 1995) from pooled samples of 30 flies from each line. Detailed comparisons of the 5′ truncations among all lines, as conducted for Figures 4 and 5, eventually led to the elimination of three lines. One line was eliminated because it contained R1 and R2 truncation profiles identical to that of another line, suggesting that it represented a contamination. Two lines were eliminated because they had higher numbers of R1 5′ truncations than other lines with many of these truncations not yet fixed in all animals. While this could represent contamination, the R2 5′ truncation profiles did not appear unusual in these two lines, suggesting it is more likely that the R1 elements in these lines had undergone recent retrotransposition events. With either interpretation these lines had to be excluded from the analysis because their 5′ truncations would not represent R1 elements originally present in the Paradise, California, population (hereafter referred to as the Paradise population).

PCR amplification, cloning, and sequencing: The 5′ junctions between the 28S rRNA gene and either R1 or R2 elements were generated by PCR amplification using a 28S gene primer upstream of the element insertion site and various R1 and R2 primers specific to locations within each element (see Figure 1 for element primer locations). For the R2 junctions, the 28S gene primer was 5′-TGCCCAGTGCTCTGAATGTC-3′, complementary to sequences beginning 80 bp upstream of the R2 insertion site. The element primer was either 5′-ATA CCCACGCAGGTTCCGC-3′, 5′-GATAGAAAATCCAACGTTC TGTCC-3′, or 5′-GGAAATCTATCGAAAGATACTAGGG-3′, primers complementary to sequences located 788 bp, 1970

bp, and 3545 bp into the R2 element, respectively. For R1 junctions, 5′-CGCGCATGAATGGATTAACG-3′, complementary to the 28S gene sequence 60 bp upstream of the R1 insertion site (and 3 bp downstream of the R2 insertion site), was used in conjunction with either 5′-AGCTCACGTACCTC GTGTAC-3′, 5′-CGCATCCATGTACCGGAGGT-3′, 5′-TTTC CCTCGACGAGAAGCAGC-3′, or 5′-GTTCCACACTGAAGG GATTAC-3′, primers complementary to sequences located 1105 bp, 2062 bp, 3728 bp, and 5293 bp into the R1 element, respectively. Because the entire sequence of the R1 element in *D. simulans* has not been determined, these distances correspond to the *D. melanogaster* R1 sequence (Jakubczak *et al.* 1990).

PCR amplifications were conducted in 1× PCR buffer (GIBCO-BRL, Gaithersburg, MD) containing 0.2 mm each dNTP, 1 mm MgCl, 0.25 μm of each primer, and 1.25 unit of Taq DNA polymerase (GIBCO-BRL). Reactions were conducted in a Perkin-Elmer Cetus DNA Thermal Cycler at a 60° annealing temperature for 28 cycles. Products were separated on 1.5% agarose gels or 8.75% polyacrylamide gels and stained with ethidium bromide. All PCR product sizes were determined relative to a combined *Hin*dIII-digested λDNA/*Hae*III-digested ϕX174 DNA standard (GIBCO-BRL).

PCR products from individual males generated using the upstream 28S gene primer and the R1 or R2 primer closest to the 5′ end (0.8 and 1.1 kb, respectively) were cloned into a modified mp18 vector as previously described (Burke *et al.* 1998). Clones were sequenced using the Universal Sequencing Primer (United States Biochemical, Cleveland) and analyzed using MacVector 6.5.3 (Oxford Molecular, Palo Alto, CA).

Genomic blot protocols: The genomic DNA blotting procedure was as described previously, except 1.2% agarose gels were used (Jakubczak *et al.* 1992). To visualize 5′ truncations of the R2 element (Figure 2), genomic DNA was digested with *Bcl*I, which cleaves the 28S gene 0.9 kb upstream and 0.35 kb downstream of the R2 insertion site, but not within the R2 element. The fragments were separated on agarose gels and probed with a 425-bp segment specific to the 3′ end of the R2 element (probe 1, Figure 1). To quantify levels of R1 and R2 insertion (Table 1), genomic DNA was digested with *Hin*dIII and *Pst*I, which cleave 3.1 kb upstream and 0.35 kb downstream of the R2 site, within the R1 element 0.9 kb from its 3′ end and within the R2 element 0.6 kb from its 3′ end. When this DNA was probed with a 280-bp segment specific to 28S sequences immediately downstream of the R1 insertion site (probe 2 in Figure 1), the uninserted, R1-inserted, and R2-inserted rDNA units gave rise to fragments of 3.4 kb, 1.2 kb, and 0.9 kb, respectively. Blots were exposed on a PhosphoImager cassette and quantified using a Storm Analyzer (Molecular Dynamics, Sunnyvale, CA) using ImageQuant 1.0. The fraction of uninserted rDNA units, R1-inserted units, and R2-inserted units was calculated by dividing the signal from each appropriate band by the total amount of signal from all three bands. However, because both R1 and R2 elements can be found in the same 28S gene, the R1 band in this blot contained rDNA units with just R1 insertions as well as units containing both R1 and R2 insertions (R1 + R2). To determine the levels of doubly inserted rDNA units, and thus the total level of R2, genomic DNA was digested with *MspA1*I and hybridized with the R2 probe (probe 1 in Figure 1). Because *MspA1*I cleaves R2 elements 1.0 kb from their 3′ end, full-length R1 elements 0.2 kb from their 5′ end, and the 28S gene 1.3 kb downstream of the R2 site, this blot gives two bands, one corresponding to doubly (R1 + R2) inserted units (1.2 kb) and the other corresponding to units containing only R2 insertions (2.3 kb). The fraction of rDNA units containing both elements (R1 + R2) was determined by first taking the ratio of R1 + R2 insertions to insertions of R2 alone. This ratio was then multiplied by the fraction of R2 determined from the original blot to give the fraction of the rDNA units containing double insertions. The fraction of doubly inserted units was then added to the singly inserted R2 units to give the total level of R2 insertions.

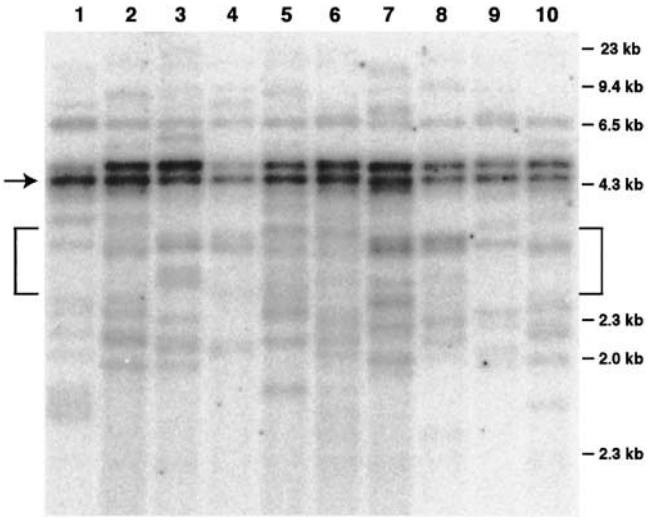

Figure 2.—Genomic blot of the R2 5′ truncation profiles. Genomic DNA from 10 lines of the Paradise population was digested with *Bcl*I, blotted, and probed with a ^{32}P-labeled 425-bp fragment specific to the 3′ end of the R2 element (probe 1 in Figure 1). *Bcl*I cleaves the 28S gene upstream and downstream of the R2 insertion site but not within the R2 element itself. Full-length R2 elements therefore give rise to a 4.5-kb band (indicated with arrow). The major band at 4.7 kb corresponds to the full-length R2 element in a rDNA unit also containing a R1 insertion (*Bcl*I cleaves a site 0.7 kb into the R1 element). Bands below the full-length element represent truncations at the 5′ end of the element. Bands migrating above the full-length element represent R2 elements in units containing a truncated R1 element. Numbers to the right correspond to the position of DNA size standards. The bracketed region corresponds to the 5′ truncations also revealed by the PCR analysis in Figure 4B. Lanes 1 through 10 correspond to strains 1, 6, 13, 37, 42, 57, 80, 89, 91, 100, respectively.

For determination of the numbers of rDNA units in the Paradise population, genomic DNA was digested with *Dra*I and blotted. *Dra*I generates a 2.6-kb restriction fragment containing the *Alcohol dehydrogenase* gene and a 1.4-kb fragment from the rDNA unit spanning the internal transcribed spacer region through the 5′ end of the 28S gene of the rDNA unit. As a measure of a single copy gene, the DNA was probed with a 1.0-kb *Adh* segment derived from the region between intron A and exon 4. After quantitation of the *Adh* signal, rDNA units were detected by probing with a 1.0-kb segment spanning the 5.8S rRNA gene and part of the 5′ end of the 28S gene (probe 3, Figure 1). Some lines could not be accurately compared to the others because an unexpected restriction polymorphism split the *Adh* hybridizing band into two fragments. Relative numbers of rDNA units were determined by taking the ratio of rDNA signal to *Adh* signal. The average rDNA:*Adh* ratio was assumed to represent 250 rDNA units, the average number of units previously found in *D. simulans* (Long and Dawid 1980; Mecheva and Semionov 1992). The rDNA:*Adh* signal ratio for an individual line was then used to estimate the number of rDNA units for each line.

RESULTS

5′ truncation profiles revealed by genomic blotting methods: The *D. simulans* population sampled corresponded to 30 isofemale lines established from a single location in Paradise, California (see MATERIALS AND METHODS). We initially selected at random 10 lines for genomic blot analysis. Genomic DNA was digested with *Bcl*I, a restriction enzyme that cleaves the 28S gene to either side of the R1 and R2 insertion sites but does not cleave the R2 element sequences. The DNA was blotted and probed with a 400-bp fragment from near the 3′ end of the R2 elements (probe 1 in Figure 1). As shown in Figure 2, this hybridization revealed two closely spaced major bands. The lower band corresponded to rDNA units containing full-length R2 element insertions. The upper band corresponded to rDNA units containing insertion of both full-length R1 and R2 insertions. (There is a *Bcl*I site 0.7 kb from the 5′ end of R1 elements.) The relative intensity of these two major bands differed between lines because of differences in the number of R2 elements, the fraction of these elements that are full length, and the number of R2 elements doubly inserted in a rDNA unit with an R1 element. In addition to these two major bands, a variable number of fainter bands were also seen in each line. On the basis of our previous analysis of Drosophila R1 and R2 elements, most of the *Bcl*I fragments that migrated below the two major bands corresponded to rDNA units with 5′ truncated R2 elements. The remaining bands corresponded to doubly inserted rDNA units in which either one or both of the R1 and R2 insertions contained 5′ truncations. The latter fragments can migrate above the major full-length bands if the 5′ truncation deletes the *Bcl*I site in the R1 insertion.

The blot in Figure 2 demonstrates two important aspects of the distribution of R2 elements in the Paradise population. First, each line contains different sets of 5′ truncations. Second, each truncation variant was present at low copy number. Unfortunately, low band resolution made it difficult to determine if certain bands of somewhat greater intensity corresponded to duplicate copies of the same 5′ truncation or multiple independent 5′ truncations of slightly different lengths. In addition, the low resolution of these genomic blots made it difficult to determine whether bands in one lane comigrated with bands from other lanes. Thus we could not accurately estimate the fraction of specific retrotransposition events (truncations) shared between lines.

The number of different 5′ truncation variants observed on these *D. simulans* chromosomes was not unexpected as an analysis of 27 geographic lines of *D. melanogaster* revealed that each population contained a large number of R1 and R2 5′ variants (see Figure 6 of JAKUBCZAK *et al.* 1992). However, it was surprising to find that different X chromosomes from the same population had such different R2 truncation profiles. Genomic blots to directly monitor R1 5′ truncations have not been conducted because R1 copies also exist in the centromeric heterochromatin of Drosophila (ROIHA *et al.* 1981; JAKUBCZAK *et al.* 1992; EICKBUSH and EICKBUSH 1995). Thus, on genomic DNA blots the only certain means to score those R1 elements that are inserted within the rDNA locus was to use 28S gene sequences as a probe. Unfortunately, such blots are complicated by the large fraction of the R1-inserted rDNA units of *D. simulans* also containing R2 insertions (see Table 1 below).

Sequence analysis of the R1 and R2 5′ junctions: To estimate more accurately the number of different R2 5′ junctions present on X chromosomes of the Paradise population, a representative sample of the 5′ junctions of full-length and near full-length R2 elements were sequenced from two of the Paradise lines (1 and 6). These junctions were obtained by PCR amplifications in which one primer annealed to the 28S gene sequences 80 bp upstream of the R2 insertion site while the second primer annealed to R2 sequences 0.8 kb from the 5′ end of full-length R2 elements. The PCR products from each line were cloned into sequencing vectors and individual clones sequenced (see MATERIALS AND METHODS).

As shown in Figure 3, the 36–38 clones sequenced from each line revealed 6 different junctions in line 1 that could be defined as full length (*i.e.*, contained no more than a 1-bp deletion of R2 sequences) and 8 junctions containing R2 5′ truncations of from 476 to 703 bp. Line 6 contained 10 different junctions that are essentially full length (*i.e.*, contained no more than an 8-bp deletion) and five truncations of from 323 to 641 bp. Because many of these junctions were found in only one or two clones, it is likely that additional junctions would be found if more clones were sequenced. Many of the full-length and 5′ truncated R2 junctions also contained deletions or duplications of the 28S target site as well as sequences not derived from either the element or target sequences. These highly variable 5′ junctions are characteristic of R2 junctions found in other species of Drosophila as well as in other arthropods (GEORGE *et al.* 1996; BURKE *et al.* 1999).

Of the different R2 5′ junctions sequenced from *D. simulans*, only 6 were found in both lines (junctions indicated with a dot to the left of the sequence in Figure 3). The 2 junctions at the top of each list in Figure 3 correspond to the most common form of Drosophila R2 insertions, a full-length R2 sequence with either one or two base pairs of the target sequence deleted (GEORGE *et al.* 1996). These results confirmed the suggestion from the genomic blot that there are a large number of R2 elements with different 5′ junctions in each line. The 14 and 15 distinctive R2 5′ junctions found in lines 1 and 6, respectively, would generate *Bcl*I fragments that would all migrate on the blot in Figure 2 at either the location of the two major bands or imme-

TABLE 1

Fraction of the rDNA units containing R1 and R2 insertions

Line	Uninserted	Total R1	Total R2	R1/R2 double		Total units[b]
				Observed	Expected	
1[a]	0.71	0.13	0.19	0.09	0.02	ND
6	0.70	0.14	0.25	0.09	0.03	255
13	0.69	0.12	0.25	0.06	0.03	ND
37	0.65	0.16	0.35	0.16	0.06	100
42	0.67	0.17	0.23	0.07	0.04	315
57	0.57	0.20	0.29	0.06	0.06	285
80	0.53	0.18	0.35	0.06	0.06	280
89	0.69	0.14	0.21	0.04	0.03	270
91	0.69	0.13	0.26	0.08	0.03	245
100	0.70	0.15	0.20	0.05	0.03	325
Average	0.66	0.15	0.26	0.08	0.04	259

ND, not determined because of a restriction polymorphism within the *Adh* gene.

[a] An extra band that accounted for 0.07 of the rDNA units was observed in this line. This band probably represents a restriction polymorphism within the R1 or R2 elements of this line.

[b] The total number of rDNA units was determined on the basis of the hybridization signal of the rDNA unit compared to a single copy gene control (*Adh*) assuming an average of 250 units in *D. simulans* (see MATERIALS AND METHODS).

diately below these bands. On the basis of this genomic blot there are clearly many more 5′ junctions in each line that involve deletions of R2 sequences >0.8 kb.

Using an approach similar to that for the R2 elements, 24 clones corresponding to R1 5′ junctions were sequenced from each of lines 1 and 6. All clones corresponded to only two types of junctions. Both junctions corresponded to full-length R1 sequences but one contained a 14-bp target site duplication of the 28S gene, while the second junction had a 21-bp deletion of the upstream 28S target site. These same junction types were observed at the 5′ end of full-length R1 elements in *D. melanogaster* (JAKUBCZAK *et al.* 1990) and is consistent with our unpublished data suggesting that full-length R1 insertions in all arthropods show less sequence variation at their 5′ junctions than R2 elements (W. BURKE and J. GEORGE, unpublished data).

PCR analysis of R1 and R2 5′ truncations: Attempting to characterize all full-length and 5′ truncated R1 and R2 elements present in the rDNA locus by sequencing would not only be tedious but still would not ensure that all junctions had been obtained. This approach would be even more laborious in a survey of multiple chromosomes in a population. We have therefore developed a more rapid PCR assay that provides an accurate minimum estimate of the number and types of variants that contain large deletions of their 5′ end. In this approach one PCR primer, complementary to the 28S gene sequences upstream of the insertion site, is used in combination with a series of second primers, complementary to different regions within the R1 or R2 element (Figure 1). This approach allowed high resolution of the 5′ truncations because it eliminated the need for the DNA blotting step and enabled all variant bands to be monitored as smaller DNA fragments. Equally important, this approach allowed 5′ truncations to be monitored independently of whether they were present in a rDNA unit that also contained another insertion.

To score all R2 5′ truncations three PCR primers that anneal to R2 sequences located 0.8 kb, 2.0 kb, and 3.6 kb from the 5′ end of a full-length element were generated (Figure 1). Similarly located PCR primers were also used for the R1 elements, as well as a fourth primer 5.3 kb from the 5′ end, due to the greater length of R1 elements. The use of multiple primers was critical in scoring all 5′ truncations on a chromosome; shorter-length PCR products dominate amplification reactions (*i.e.*, those copies with 5′ truncations that extend to near the primer binding site), thus leading to an underscoring of 5′ truncations that end farther upstream of the primer binding site.

Examples of the PCR products that were generated using the 2.0-kb R1 and R2 primers are shown in Figure 4. In Figure 4A, the many full-length R1 elements in each Paradise line were represented by the major amplification band at 2.1 kb, while the shorter-length products represented the one to four 5′ truncated R1 elements in each line revealed by this primer combination. Of the various truncated copies scored on this gel, one gave rise to a 1.7-kb band observed in all lines, a second gave rise to a 1.0-kb band present in four lines, while five were unique to individual lines. These truncated bands varied in intensity due to differences in their length and the presence of amplifying bands of lower size. In Figure 4B, the larger number of 5′ truncated R2 products has been separated on a higher resolution polyacrylamide gel. Only the region of the gel corresponding to fragments from 0.1 to 0.8 kb in length is

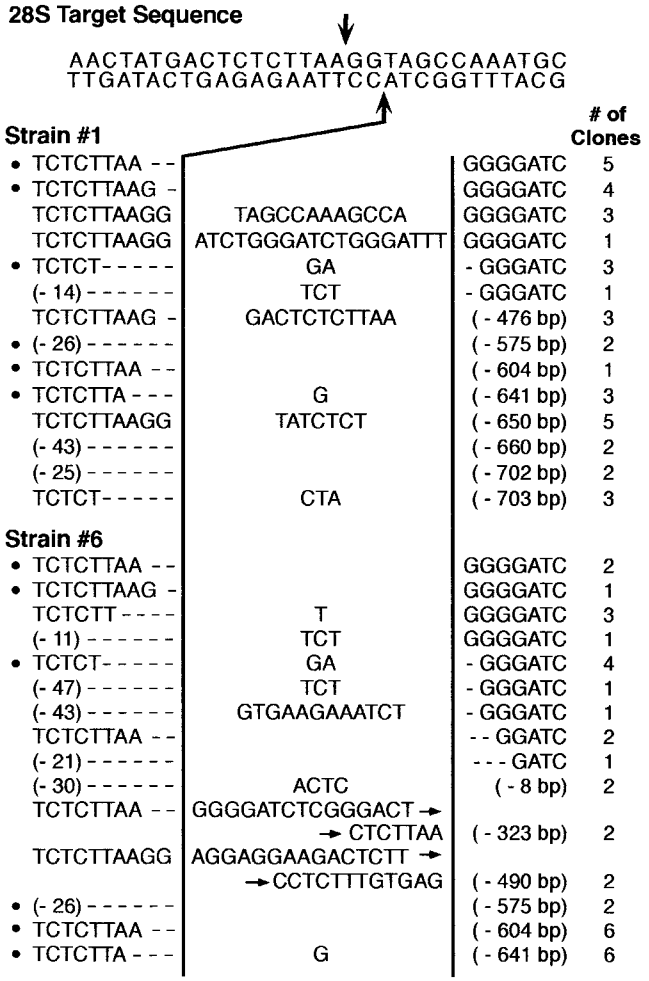

FIGURE 3.—The 5′ junction sequences of full-length and near full-length R2 elements from two strains of the Paradise population. Both strands of the 28S gene sequence at the R2 insertion site are shown at the top of the figure with the location of the R2 endonuclease cleavage site on each strand indicated with an arrow. Below this sequence are the sequences of the 5′ junctions of 40 clones obtained by PCR. Nucleotides to the left of both vertical lines represent upstream 28S gene sequence, nucleotides to the right of both vertical lines represent R2 sequences, while nucleotides between the two vertical lines represent duplications of portions of the R2 element, the 28S gene, or nontemplate sequences that are sometimes inserted during retrotransposition. Dots next to some sequences denote junctions that are shared between the two lines.

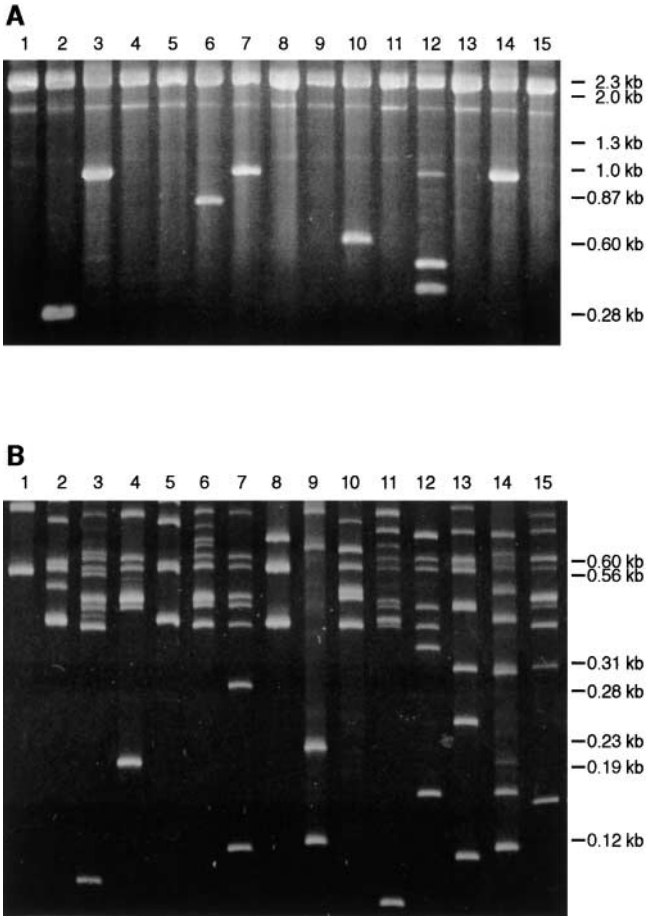

FIGURE 4.—The 5′ junctions revealed by the PCR assay. In A and B, one PCR primer annealed to the 28S gene upstream of the insertion site, while the second primer annealed to the element 2.0 kb from its 5′ end. (A) R1 5′ junctions separated on a 1.2% agarose gel. Lanes 1 through 15 correspond to lines 58, 61, 65, 71, 76, 80, 84, 88, 89, 90, 91, 92, 95, 96, and 100, respectively. (B) R2 5′ junctions separated on an 8% polyacrylamide gel. Lanes 1 through 15 correspond to strains 4, 6, 9, 13, 16, 20, 31, 32, 34, 37, 39, 42, 45, 59, and 61, respectively. In A and B, numbers to the right correspond to the position of DNA size standards.

shown in the Figure 4. The larger number of truncated R2 elements that could be scored by this PCR approach compared to the genomic blot is readily seen by a comparison of Figure 4B with the bracketed region of the genomic blot in Figure 2. As was found for the R1 elements, some of the 2–10 R2 elements with 5′ truncations that ended within this 0.7-kb region appear to be present in multiple lines of the Paradise population, while other truncations are unique to specific lines.

What is the total number of R1 and R2 5′ truncations and what fraction of these variants is shared between different chromosomes within the Paradise population? In the case of R2, accurate estimates of the number of 5′ variants that are shared by different chromosomes in the Paradise population are complicated by the many PCR bands of similar length. In particular, there appear to be preferred positions along the R2 element where truncations are likely to occur. For example, there are a large number of R2 truncations from 0.5 to 0.7 kb in length (see sequence analysis in Figure 3) and from 1.3 to 1.5 kb in length (the many 0.4- to 0.6-kb bands in Figure 4B). Therefore, in Figure 5 we have simply scored the total number of different R2 5′ variants in each Paradise line generated by this PCR approach (column on right side). Each line was found to have from 12 to 34 clearly resolved 5′ truncated bands with an average of 21 bands. These values should be considered a mini-

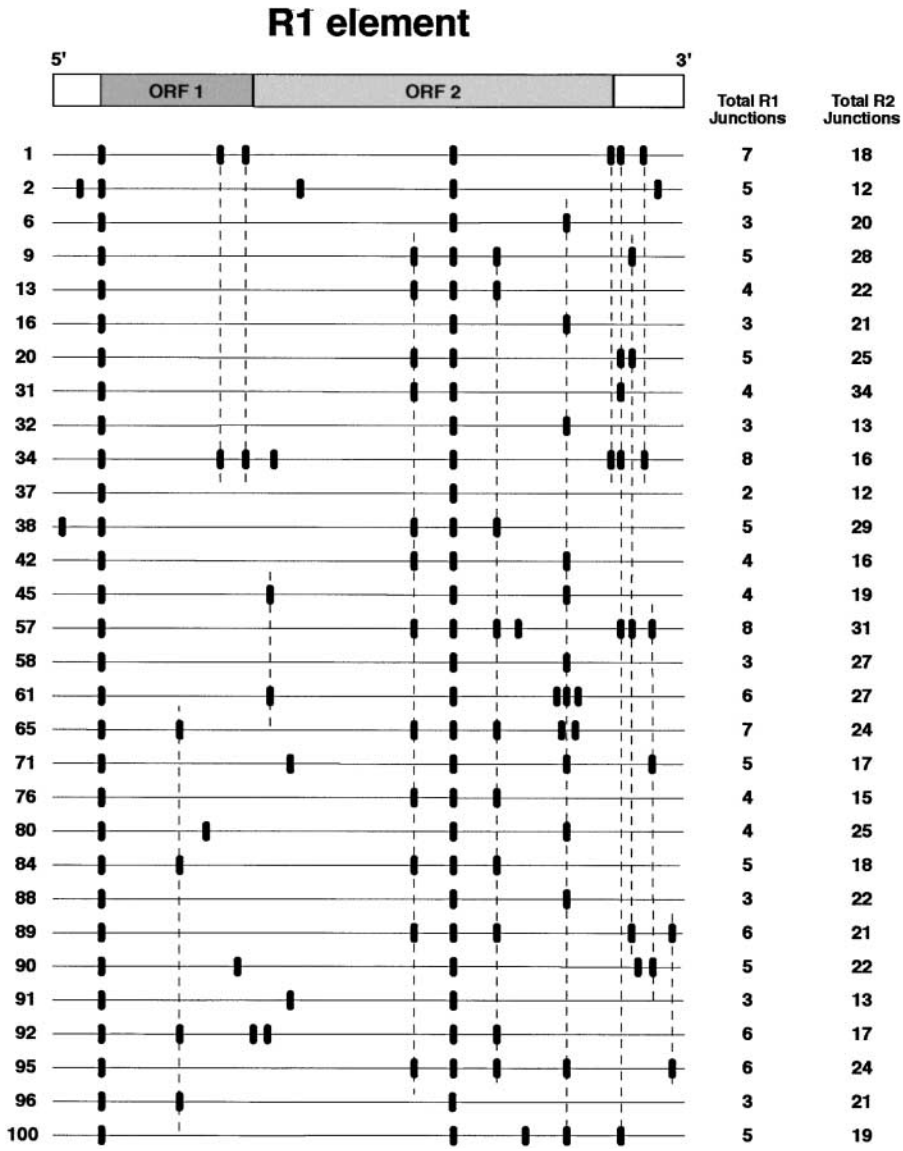

FIGURE 5.—Graphic representation of the position and distribution of the R1 5′ truncations in all 30 Paradise lines. Shown at the top is a diagram of the R1 element. The approximate length of the 5′ truncations in each line is indicated by the solid vertical lines on the thin horizontal lines. Dashed lines connecting the solid vertical lines in two or more lines represent the same length variant on different lines. The Paradise line numbers are given at left. To the right are two columns representing the total number of different 5′ truncation variants present in the R1 and R2 elements of that line.

mum estimate, however, as certain PCR-amplified bands were of greater intensity than those bands located immediately above and below. These more intense bands could have represented multiple copies of a specific truncation or different 5′ truncations that gave rise to PCR fragments of similar length. For example, because of differences in the size of the 28S gene deletions and the number of extra nucleotides, the R2 5′ variants with deletions of 641 and 650 bp in line 1 (see Figure 3) will generate PCR products of identical length.

In the case of the R1 elements, each X chromosome in the Paradise population generated a total of only two to eight 5′ truncated bands that were more or less randomly distributed over the 5.3-kb length of the elements. The fewer numbers of R1 variants suggested we could accurately determine the total number of different 5′ truncations in each line and how widely each of these variants was shared between the different Paradise lines. A summary of this data is shown in Figure 5. The length of each R1 5′ truncation is shown by the thick vertical bar on the horizontal thin line representing the length of a complete element. The 5′ truncated elements that are of the same length in different lines of the Paradise population are connected by a thin, dashed line.

Plotted in Figure 6 is the degree to which the 33 different-length R1 5′ variants that were scored in the Paradise population were shared between the 30 X chromosomes sampled. Nineteen of these truncations (58%) were present in only 1 line, while 6 (19%) were shared by 2 lines. It should be noted that 4 of these shared variants are represented by lines 1 and 34, suggesting that our collection of 30 lines sampled this chromosome twice. Only 8 of the different truncations (24%) were shared by 3 or more lines. Thus only a small fraction of the R1 variants represent older insertions that are distributed to a significant fraction of the X chromosomes in the population.

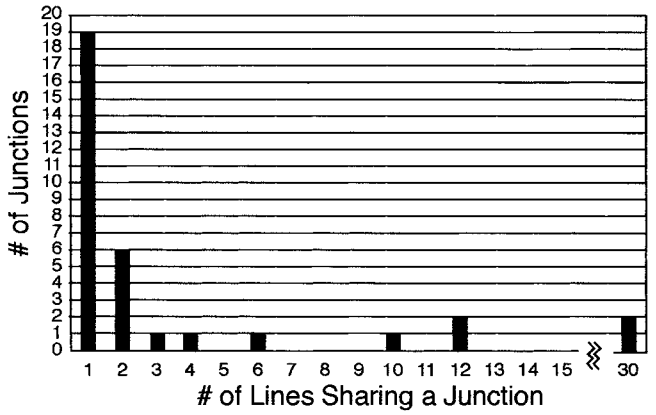

FIGURE 6.—Most R1 5′ variants in the Paradise population are unique to individual chromosomes. The data are the same as that shown in Figure 5 but plotted as a graph. A total of 33 different 5′ variants were found in the Paradise chromosomes. Only 2 of these variants were fixed in all 30 chromosomes tested from this population. A majority of the variants were unique to individual chromosomes.

Two R1 variants were found in all lines. The PCR products from lines 1 and 6 corresponding to the longer of the two variants were excised from a gel, cloned, and sequenced. The sequence from both lines revealed that this variant had a large internal deletion within the first open reading frame of the element (data not shown). Such internal deletions have not been found in R1 and R2 elements within the rDNA locus of Drosophila species (EICKBUSH and EICKBUSH 1995; LATHE *et al.* 1995; LATHE and EICKBUSH 1997; GENTILE *et al.* 2001). However, defective R1 elements have been found outside the rDNA locus in heterochromatic regions (KIDD and GLOVER 1980; ROIHA *et al.* 1981; EICKBUSH and EICKBUSH 1995). Internal deletions are common in copies of mobile elements located in heterochromatic regions that are undergoing mutation and gradual DNA elimination (PETROV *et al.* 1996). While previous sequence and genomic blot analyses have shown that there are very few R1 copies located outside the rDNA units of *D. simulans* (EICKBUSH and EICKBUSH 1995), it is possible that one, or even both, of the fixed R1 5′ truncations observed in the Paradise lines may represent such non-rDNA copies.

Levels of R1 and R2 insertion in the Paradise population: The different patterns of 5′ variants detected on each chromosome in the Paradise population suggested that multiple R1 and R2 retrotransposition events had occurred on each chromosome. Do the absolute levels of R1 and R2 also vary in this population? How much has recombination changed the total number of rDNA units on these chromosomes? A series of genomic blots was conducted to determine the percentage of R1 and R2 insertions on individual chromosomes of the Paradise population, what percentage of these insertions corresponded to double (R1 + R2) insertions, and finally to estimate the total number of rDNA units on each chromosome (see MATERIALS AND METHODS for a description of these genomic blots). The Paradise lines used for this analysis were the same as those used in Figure 2. The results are summarized in Table 1.

The fraction of the rDNA units in these Paradise lines inserted with R1 and R2 varied almost twofold (range 0.12–0.20 for R1 and 0.19–0.35 for R2). Meanwhile, the absolute number of rDNA units varied threefold. This wider range in the number of rDNA units was a result of line 37, which was estimated to have only ~100 rDNA units. The remaining lines had an estimated 245–325 units. It is interesting to note that only ~65 units in line 37 are uninserted with either R1 or R2, which is near the minimum number of rDNA units needed to avoid the *bobbed* phenotype in *D. melanogaster* (HAWLEY and MARCUS 1989). Line 37 males and females did not show obvious signs of the *bobbed* phenotype.

The total number of R2 elements within different rDNA loci of the Paradise population ranged from 35 to 98, which was about twice the number of R1 elements (range 16 to 57). R2 elements appear to undergo 5′ truncations more frequently as there are, on average, nearly four times the number of 5′ truncated R2 elements compared to R1 (Figure 5). As would be expected, the lowest total number of R1 and R2 elements, as well as the lowest number of R1 and R2 5′ truncation variants, were in line 37, the line that appears to have undergone a large deletion within its rDNA locus.

Finally, a surprisingly high fraction of rDNA units in the Paradise population contained both an R1 and R2 insertion. Assuming random insertion of R1 and R2 elements, the fraction of units containing both insertions is simply the fraction of the units with an R1 insertion multiplied by the fraction of units with an R2 insertion. As shown in Table 1, almost all of the lines in the Paradise population contained an excess of these double insertions. In one case, line 37, essentially all R1 elements are doubly inserted with R2 elements. A two-tailed *t*-test of the expected *vs.* the observed values indicates that these differences are significant ($t = 2.18$, $P = 0.0098$). This result differs from that of JAKUBCZAK *et al.* (1992), who found no significant difference between the observed and expected percentage of double insertion across strains of *D. melanogaster*. This difference could reflect species-specific differences in the insertion properties of the R1 and R2 elements or in their rate of turnover. Alternatively, this difference could be related to the higher levels of R1 insertions in most *D. melanogaster* strains or because the rDNA units in *D. melanogaster* are located on both the X and Y chromosomes.

DISCUSSION

R1 and R2 elements are abundant components of arthropod rDNA loci and have been vertically inherited, possibly since the origin of the phylum (BURKE *et al.* 1998, 1999). Production of functional 28S rRNA by the

unit is eliminated when R1 or R2 elements insert, suggesting that these insertions have a deleterious effect on the organism. Indeed, high levels of R1 and R2 insertion are associated with the *abnormal abdomen* phenotype in *D. mercatorum* (Templeton *et al.* 1989; Malik and Eickbush 1999) and the *bobbed* phenotype in *D. melanogaster* and *D. hydei* (Franz and Kunz 1981).

Extensive studies have been conducted of sequence variation within the rDNA array of *D. melanogaster* as a means to understand the concerted evolution of this locus (Linares *et al.* 1994; Schlötterer and Tautz 1994; Bowen and Dover 1995; Polanco *et al.* 1998, 2000). Unfortunately, these studies focused on variation in the internal and external spacer regions of the repeat, largely ignoring the presence of the R1 and R2 insertions. Thus little is known of the dynamics of R1 and R2 insertions. Theoretically, the recombinations responsible for the concerted evolution of the rDNA locus can either duplicate or eliminate individual copies of R1 and R2, thereby variously expanding and reducing the total number of elements. The large variation we have observed in the fraction of rDNA units inserted with R1 and R2 appears consistent with such expansion and contraction events (Jakubczak *et al.* 1992). Furthermore, the very high sequence identity of these elements within a species (>99% at the nucleotide level) also appears consistent with the forces of concerted evolution (Eickbush and Eickbush 1995; Lathe *et al.* 1995; Lathe and Eickbush 1997).

Because R1 and R2 cannot become fixed in all rDNA units of the host, the long-term net effect of the concerted evolution mechanisms should be their elimination. Thus, unless these elements have found a means to avoid or manipulate the forces of concerted evolution, prolonged survival of the elements would require a counterbalancing influx of new retrotransposition events. Prior to this article there were no available data to suggest how often these elements retrotranspose. Indeed, Clark and co-workers have shown that in a population of *D. melanogaster* the variation in total number of rDNA units as well as the levels of R1 and R2 could be effectively modeled as a simple function of recombination (Lyckegaard and Clark 1991). Thus there was no need to factor in retrotransposition to explain this variation.

We have shown in this study that a simple determination of the number of R1 or R2 insertions significantly underestimates the rate of turnover of these elements in a population. While the level of R1 and R2 insertions varied less than twofold, each X chromosome of the Paradise population was found to have a unique collection of 5′ truncated R1 and R2 elements. Because these 5′ truncations are generated by the TPRT mechanism used for integration, each chromosome in the population contained a unique set of retrotransposition events. A majority of these 5′ variants was found on individual chromosomes, suggesting that most integration events were sufficiently recent that they have not spread by recombination to other chromosomes in the population.

Even more revealing was the total number of different R1 and R2 5′ variants on the same chromosome. Population analysis of the rDNA locus has suggested that intrachromosomal recombinations spread sequence variation within the ITS region to the other rDNA units on the same chromosome more rapidly than they do to other chromosomes in the population (Schlötterer and Tautz 1994; Polanco *et al.* 1998, 2000). Each chromosome in the Paradise population had 2–8 different 5′ truncated R1 elements and 12–34 different 5′ truncated R2 elements. None of these 5′ variants had expanded to high copy number within the rDNA locus of any chromosome. In the case of R2, our estimates of the number of elements present on each chromosome (35–98 elements) and the fraction of these elements that contained 5′ truncations (<50%) indicate that the 12–34 different 5′ truncations observed on each chromosome were present at one or at most a few copies per rDNA locus. The similar intensity of the different variant bands on both the genomic blots (Figure 2) and the PCR amplifications (Figure 4) would further suggest that most of the variants are present at only one copy per rDNA locus. The Paradise population is not unusual in this regard. Preliminary observations have indicated similar high numbers of R1 and R2 5′ truncations, with each variant present at essentially one copy in the rDNA locus in other strains of both *D. melanogaster* and *D. simulans* (C. Pérez-González and D. Eickbush, unpublished data).

Evolution of R1 and R2 within a population: Our analysis of the Paradise population indicates that individual copies of the R1 and R2 elements are not being expanded (duplicated) by recombination to additional units in the same rDNA locus. This in turn suggests that the recombinational mechanism(s) responsible for spreading sequence variants between rDNA units on the same chromosome is (are) biased against R1 and R2 insertions. Of the two mechanisms usually considered responsible for the concerted evolution of the rDNA locus, gene conversion and unequal crossovers, only gene conversion has been suggested to have a bias. A gene conversion bias against some but not all insertion sequences has been reported in yeast (see discussions in Holliday 1982; Vincent and Petes 1989), and a bias against certain rDNA units has been noted in some interspecies crosses (Hillis *et al.* 1991). Thus a recombinational bias against R1 and R2 is perhaps not unexpected. What is surprising is that without a means to overcome this bias R1 and R2 have remained so successful in so many different arthropod lineages.

If the homogenization of rDNA units on one chromosome is faster than the homogenization of units between chromosomes (Schlötterer and Tautz 1994), those 5′ variants that have spread to other chromosomes in the population should be the oldest and thus have had

ample opportunity to expand to other units on the same chromosome. However, the R1 or R2 variants that were found on multiple chromosomes in the Paradise population were no more abundant than those variants that are present on only one chromosome. This apparent contradiction could be explained if there are locations in the rDNA locus that are not as frequently subjected to intrachromosomal recombinations but are readily subjected to interchromosomal recombinations. One such location would be the rDNA units that are located at the ends of the long tandem arrays. Terminal units have been shown to accumulate the most sequence variants in tandem arrays, suggesting that they are less frequently subjected to the homogenizing forces of recombination within the array (McAllister and Werren 1999). On the other hand, terminal repeats are readily exchanged between different chromosomes in a population by simple crossover events. One would thus predict that R1 and R2 elements would accumulate at the ends of a rDNA array and that these are the insertions most frequently spread to other chromosomes in a population. Consistent with this model, *in situ* hybridization data have indicated that R1 and R2 elements are concentrated at the edges of the rDNA array in *Bombyx mori* (H. Maekawa, personal communication).

Evolution of R1 and R2 within a species lineage: The rapid turnover of R1 and R2 elements in the rDNA locus revealed by this study helps to explain a confusing issue we have encountered in our analysis of the evolution of arthropod R1 and R2 elements. We have found that many arthropod species contain more than one family of R1 or R2 elements (Burke *et al.* 1993, 1998; Gentile *et al.* 2001). Sequence divergence of elements from the same family is low (<1% at the nucleotide level) while sequence divergence between families is high (>30% nucleotide divergence). We originally explained divergent families of elements in the same species as likely examples of horizontal transmission (Burke *et al.* 1993). However, our continuing analysis of these families, in particular a detailed analysis of multiple R1 families in Drosophila, has provided no evidence for horizontal transmissions (Gentile *et al.* 2001). The problem thus became how two families of elements could arise *de novo* within a species, because we assumed concerted evolution maintained the sequence identity of the family.

The analysis of the rapid turnover of R1 and R2 elements in the Paradise population suggests that the high level of sequence identity observed is not a result of the process of concerted evolution. Rather, it is the result of the rapid turnover of the elements within each locus and thus the preservation of a small pool of active copies in each species. This model also predicts that a large fraction of the R1 and R2 elements in any species are recent insertions and thus should contain intact open reading frames. This is precisely what we have encountered in our sequencing of both full-length and 5′ truncated elements from many arthropods (Eickbush and Eickbush 1995; Lathe *et al.* 1995; Burke *et al.* 1998).

In the absence of concerted evolution, multiple lineages of R1 and R2 elements can evolve in a manner similar to mobile elements that insert at locations throughout the host genome. That is, the progeny of every active element accumulate sequence changes independently of each other. Eventually these progeny can accumulate sufficient sequence differences that they become distinct lineages. Of course, in the case of R1 and R2, the limited number of rDNA units in each species would mean these lineages are in competition for a limited number of insertion sites. As a result, it is unlikely that a large number of R1 and R2 lineages could be simultaneously maintained in a species. Consistent with this suggestion, while four R1 lineages have been detected in the Drosophila genus, most species contain a single lineage and no species contains more than two lineages (Gentile *et al.* 2001). We have, however, identified a few insects (*e.g.*, Japanese beetle, parasitic wasp) that contain as many as five different lineages (Burke *et al.* 1993). Perhaps these species have highly divided population structures that permit more lineages to coexist in the same species.

In summary, the data in this article strongly suggest a rapid rate of retrotransposition and elimination of R1 and R2 elements in Drosophila. However, because the Paradise lines were derived from females whose genetic relationships are unknown, absolute retrotransposition and elimination rates for the R1 and R2 elements cannot be determined. To obtain such direct estimates would require a monitoring of these events over time in individual lines. To this end we have initiated a survey of the Harwich mutation accumulation lines of *D. melanogaster* developed by Mackay *et al.* (1992). These lines have been used previously to estimate rates of transposition for mobile elements in *D. melanogaster* (Nuzhdin and Mackay 1994, 1995). Our preliminary analysis suggests that both new R1 and R2 retrotransposition and elimination events can be readily scored in these lines, which should allow direct estimates of these critical rates (C. Pérez-González, unpublished data).

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