Rates of R1 and R2 Retrotransposition and Elimination From the rDNA Locus of *Drosophila melanogaster*

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Manuscript received April 3, 2002
Accepted for publication July 8, 2002

ABSTRACT

R1 and R2 elements are non-LTR retrotransposons that insert specifically into the 28S rRNA genes of arthropods. The process of concerted evolution of the rDNA locus should give rise to rapid turnover of these mobile elements compared to elements that insert at sites throughout a genome. To estimate the rate of R1 and R2 turnover we have examined the insertion of new elements and elimination of old elements in the Harwich mutation accumulation lines of *Drosophila melanogaster*, a set of inbred lines maintained for >350 generations. Nearly 300 new insertion and elimination events were observed in the 19 Harwich lines. The retrotransposition rate for R1 was 18 times higher than the retrotransposition rate for R2. Both rates were within the range previously found for retrotransposons that insert outside the rDNA loci in *D. melanogaster*. The elimination rates of R1 and R2 from the rDNA locus were similar to each other but over two orders of magnitude higher than that found for other retrotransposons. The high rates of R1 and R2 elimination from the rDNA locus confirm that these elements must maintain relatively high rates of retrotransposition to ensure their continued presence in this locus.

R1 and R2 are non-LTR retrotransposable elements that insert specifically into the 28S rRNA genes in the rDNA loci of arthropods (Jakubczak *et al.* 1991; Burke *et al.* 1993, 1998). Phylogenetic analyses of these elements in the genus Drosophila indicate a strictly vertical transmission of R1 and R2 (Lathe *et al.* 1995; Lathe and Eickbush 1997; Gentile *et al.* 2001). Further analysis of diverse arthropods suggests these elements may have been vertically transmitted since the origin of the phylum >600 million years ago (Burke *et al.* 1998; Malik *et al.* 1999). Thus, R1 and R2 have been remarkably stable despite their deleterious effect on individuals through the reduction of 28S rRNA transcription (Kidd and Glover 1981; Jamrich and Miller 1984).

The tandemly repeated rRNA genes undergo concerted evolution driven by gene conversion and unequal crossing over (Coen *et al.* 1982; Lyckegaard and Clark 1991; Schlötterer and Tautz 1994; Polanco *et al.* 1998). These processes lead to the turnover of individual rDNA units and presumably of the R1 and R2 insertions in those units. We can monitor the turnover of R1 and R2 insertions, even though all copies insert into the same sequences, because of the imprecise mechanism of non-LTR retrotransposition. First, non-LTR retrotransposons are frequently 5′ truncated during the process of target prime reverse transcription. Indeed, it is not unusual for most of the copies of a non-LTR retrotransposon family to contain these 5′ truncations (Eickbush 2002; Moran and Gilbert 2002). Presumably these truncations occur when the reverse transcriptase fails to reach the 5′ end of the RNA template (Luan *et al.* 1993). Second, even when the reverse transcriptase does reach the 5′ end of the RNA template, small deletions and duplications of the element or target site frequently result, giving rise to unique 5′ junctions.

Previously, 5′ junction patterns have been used to monitor the activity of R1 and R2 elements. Different sets of 5′ length variants were found for both elements from various geographical isolates of *Drosophila melanogaster* (Jakubczak *et al.* 1992). This result suggested that R1 and R2 elements were actively retrotransposing at the species level. A more recent study of a collection of *D. simulans* isofemale lines from the same population again found different patterns of 5′ truncations for both R1 and R2 elements. This result indicated that R1 and R2 element turnover was sufficiently high to be monitored at the population level (Pérez-González and Eickbush 2001). Unfortunately absolute rates of insertion and elimination could not be determined in this population study because the isofemale lines had unknown genetic relatedness.

The best means to determine the rate of turnover for R1 and R2 in the rDNA array is to monitor identical lines maintained over known periods of time. The Harwich mutation accumulation lines of *D. melanogaster* (Mackay *et al.* 1992) meet this requirement. These lines were created by intensive inbreeding of the original Harwich strain (Kidwell *et al.* 1977). After the forty-first generation of inbreeding, individual pairs were used to establish multiple sublines, which were then maintained by mass mating of 10 pairs. These lines were originally

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created to determine the rates and properties of mutations affecting quantitative traits (Mackay *et al.* 1992, 1994). They have also been used to study the response to selection on different loci (Fry *et al.* 1995; Mackay and Fry 1996) and for the determination of the rate of insertion and excision of different transposable element families (Nuzhdin and Mackay 1994, 1995). In this report we show that these lines can also be used to determine the rates of R1 and R2 retrotransposition and elimination from the rDNA locus.

MATERIALS AND METHODS

Fly stocks and DNA isolation: Thirty Harwich lines were a kind gift of T. F. C. Mackay. Line designations were as in Mackay *et al.* (1992). Genomic DNA was isolated from individual males using the protocol of Chia *et al.* (1985), except that 50-μl volumes were used for homogenization, and after RNase A treatment the aqueous layer was extracted with an equal volume of phenol:chloroform and an equal volume of chloroform and then ethanol precipitated before resuspension in 10 mm Tris-HCl, 1 mm EDTA buffer (pH 7.5).

PCR amplification, cloning, and sequencing: DNA fragments representing the 5′ junctions between the 28S rRNA gene and either R1 or R2 elements were generated by PCR amplification, using a 28S gene oligonucleotide primer located either 73 bp (R1) or 80 bp (R2) upstream of each element's insertion site and various R1 and R2 oligonucleotide primers specific to locations within each element (see Figure 1). The sequence of each primer and its location relative to the published sequence of R1 (Jakubczak *et al.* 1990), R2 (Jakubczak *et al.* 1990), and the rDNA unit (Tautz *et al.* 1988) are given in Table 1.

PCR amplifications were conducted in 20 mm Tris-HCl (pH 8.4), 50 mm KCl, 0.2 mm dNTPs, 1 mm $MgCl_2$, 0.25 μm of each primer, and 1.25 units of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD). Reactions were conducted in a Perkin Elmer-Cetus (Norwalk, CT) DNA thermal cycler as follows: 1 min at 97°, 2 min at 55°, 3 min at 72° for 2 cycles; 1 min at 94°, 1 min at 60°, 3 min at 72° for 28 cycles; and 1 min at 94°, 1 min at 60°, 10 min at 72° for 1 cycle. PCR products were separated on native 8.75% polyacrylamide gels at 4° and stained in 45 mm Tris-Borate, 1 mm EDTA (TBE) buffer containing 12.5 μg/μl ethidium bromide. All PCR product sizes were determined relative to a combined *Hin*dIII-digested λDNA/*Hae*III-digested ϕX174 DNA standard (GIBCO-BRL).

To obtain better resolution of full-length R1 and R2 junctions, as well as 5′ truncated R1 elements ~0.5 kb in length, the R1 180-bp, the R1 5.3-kb, and the R2 150-bp primers were end labeled and the PCR products were separated on high-voltage denaturing 8% polyacrylamide gels. Labeling reactions were performed in 20 μl final volume containing 100 mm Tris-HCl (pH 7.6), 10 mm $MgCl_2$, 5 mm dithiothreitol, 0.1 mm spermidine, 0.1 mm EDTA, 6 pm DNA primer, 25 μCi of [γ-^{32}P]dATP (New England Nuclear, Boston), and 10 units T4 polynucleotide kinase (MBI-Fermentas). After a 60-min incubation at 37°, the enzyme was inactivated by boiling at 96°, and the primer was collected by ethanol precipitation. The end-labeled primer was then used in PCR amplification as above.

To sequence the 5′ junctions of full-length R1 elements, PCR products from line 22 were generated using the upstream 28S gene and R1 180-bp primers and cloned into a modified mp18 vector as previously described (Burke *et al.* 1995). Clones were sequenced using the universal sequencing primer (United States Biochemical, Cleveland) and analyzed using MacVector 6.5.3 (Oxford Molecular Group).

Copy number determination: Because virtually all of the PCR-amplified bands corresponding to the 5′ truncated copies of R1 and R2 were of similar intensity, these bands were interpreted as representing junctions present at one copy per haploid genome. The total number of 5′ truncated copies of R1 and R2 elements was therefore estimated by simply counting the number of bands visible with the complete range of PCR primers. In the case of the full-length elements, multiple copies gave rise to PCR products of the same length. The number of full-length copies of R1 and R2 was estimated using end-labeled R1 180-bp and R2 150-bp primers in combination with the 28S primer. The amplified products were separated on high-voltage denaturing 8% polyacrylamide gels, exposed to a PhosphorImager cassette, and the relative intensity of bands was quantified using a Storm analyzer (Molecular Dynamics, Sunnyvale, CA). The amount of radioactive signal for each band was calculated using ImageQuant 1.2 (Molecular Dynamics). Bands with the lowest levels of signal were assumed to be single copy, while the copy number represented by the more intense bands in each line was determined by dividing their intensity by the average of the single-copy signals in that line.

RESULTS

Assay of R1 and R2 5′ truncations in the Harwich lines: In previous studies we have used Southern blotting approaches, sequencing of cloned fragments, and gel analysis of PCR products to compare the 5′ junction profiles of different Drosophila strains (Jakubczak *et al.* 1992; George *et al.* 1996; Pérez-González and Eickbush 2001). The direct observation of PCR product lengths, as originally developed for the analysis of a *D. simulans* population (Pérez-González and Eickbush 2001), is the preferred approach as it enables multiple testing of single animals and provides sufficient resolution to score individual insertion and elimination events. In this approach a primer that anneals to the 28S sequence ~75 bp upstream of each element's insertion site is used in combination with a series of second primers that anneal to sequences throughout the element itself. The primers used were distributed over each element on the basis of the number of 5′ truncations in the area. The use of multiple primers enabled each junction to be confirmed with more than one primer and for each band to be maximally resolved as a low-molecular-weight product (Figure 1, Table 1). PCR products were separated on native 8.75% polyacrylamide gels, which allowed resolution of bands in adjacent lanes that differed by 5 bp or more. These initial PCR reactions enabled the unambiguous scoring of all the R2 5′ truncations and most of the R1 5′ truncations. However, there remained too many similar length truncations in the region from 4.8 to 5.0 kb from the 5′ end of the R1 elements to be resolved with the native polyacrylamide gels. This accumulation of highly truncated R1 elements only 0.5 kb in length was noted in early characterizations of type I (R1) insertions in *D.*

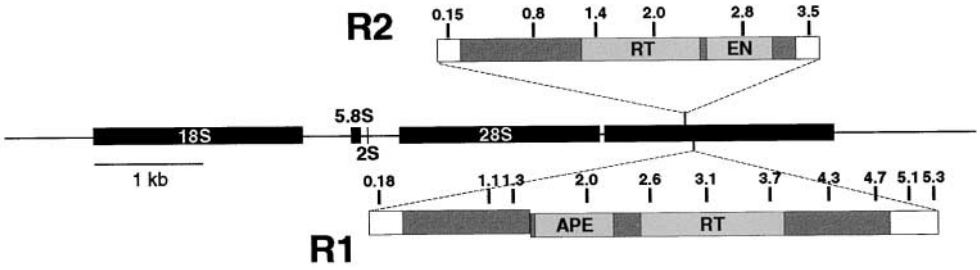

FIGURE 1.—Organization of R1 and R2 elements in the rDNA loci of *D. melanogaster*. Shown is the organization of a typical rDNA unit containing 18S, 5.8S, 2S, and 28S rRNA genes (solid boxes) separated by internal transcribed spacers. R1 and R2 elements insert into specific sites located 74 bp apart in the 28S gene. 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 locations of apurinic/apyrimidic endonuclease (APE), reverse transcriptase (RT), and C-terminal endonuclease (EN) domains are shown in lighter shading. Primer locations used in this study are denoted by a small vertical line with numbers corresponding to their location in kilobases from the 5′ end of the element.

melanogaster (WELLAUER and DAWID 1977). We therefore devised a higher-resolution assay in which the 5.1 primer was end labeled with ^{32}P, and the resultant PCR products were separated on denaturing polyacrylamide gels typically used for DNA sequencing. The 1-bp resolution provided by these gels allowed most of these highly truncated R1 elements to be viewed as different-length PCR products.

A number of the original Harwich lines had been selected for high and low abdominal and sternoplural bristle numbers (MACKAY *et al.* 1994; FRY *et al.* 1995; MACKAY and FRY 1996). We noted in our initial screens that most of the lines selected for low bristle numbers had only one-third the number of R1 and R2 elements seen in the unselected lines (data not shown). Because one of the loci affecting abdominal bristles is the rDNA locus (known in this regard as the *bobbed* locus; RITOSSA *et al.* 1966), deletions or expansions within the rDNA locus appear to have been selected in these lines. Therefore all selection lines were excluded from our analysis. The 19 unselected Harwich lines still available from the original study were examined for this study 353 generations after they were established.

Initially several males were screened from each line to determine if there was significant heterogeneity in the pattern of R1 and R2 5′ truncations. Males were used

TABLE 1

Primers used in this study

Primer	Sequence	Location[a]
Forward		
rDNA		
R2 28S gene	TGCCCAGTGCTCTGAATGTC	5833–5852
R1 28S gene	AGCCAAATGCCTCGTCATC	5915–5933
Reverse		
R2		
150 bp	GTTTAGCATTACCGGGACCAC	145–165
789 bp	ATACCCGCGTAGTGTCCGC	789–807
1.4 kb	TATTGGACGAAAGTCTTGCGG	1414–1434
2.0 kb	GATAGAAAATCCAACGTCTTGTCC	1971–1994
2.8 kb	GTGCCTTCCCCTTGTAGTACG	2785–2805
3.5 kb	GGAAATCTTCGAAAGATACTAGGT	3548–3571
R1		
180 bp	GCTCCGCCGCTCAACACGTGC	176–196
1.1 kb	AGCTCACGTACCTCGTGTAC	1105–1124
1.3 kb	TGGTCACCAGGTGCACCGACT	1352–1372
2.0 kb	CGCATCCATGTACCGGAGGT	2062–2081
2.6 kb	GAGTCAACTTCCTTCCTAGCG	2596–2616
3.1 kb	AGATAGTGCCATTGATGCCGT	3091–3111
3.7 kb	TTTCCCTCGACGAGAAGCAGC	3728–3748
4.3 kb	CGTAAAGCCAGTCGTTCTCCT	4318–4338
4.7 kb	GCCGTTGAGTCTTCTCTTGAT	4726–4746
5.1 kb	GTCACAGCACCCGAGTTTTGC	5077–5097
5.3 kb	GTTCCACACTGAAGGGATTAC	5293–5313

[a] Nucleotide positions based on the published sequences: rRNA unit, accession no. M21017; R2, X51967; and R1, X51968.

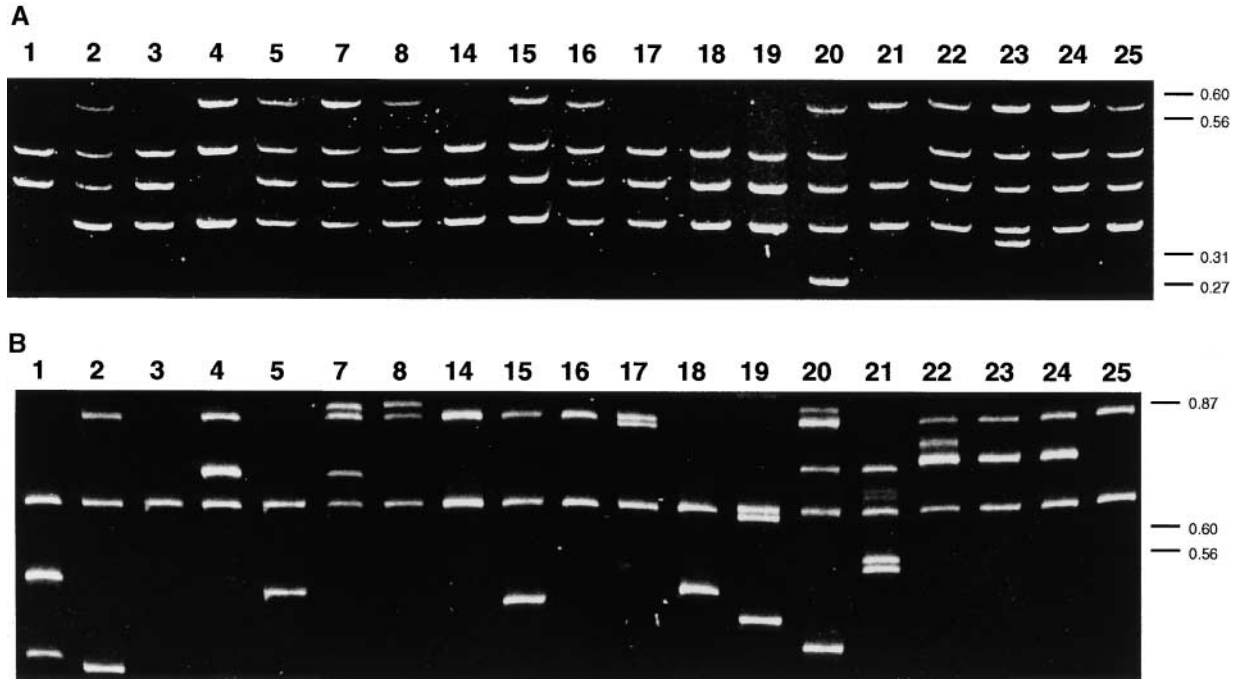

FIGURE 2.—PCR assay to determine the 5′ truncation profiles of R1 and R2 elements. (A and B) One PCR primer annealed to the 28S gene upstream of the insertion site, while the second primer annealed to the element at some distance downstream from the 5′ end. Numbers at the top correspond to the Harwich line number, while numbers to the right correspond to the position of DNA size standards. The PCR products were separated on 8.25% polyacrylamide gels. (A) R2 5′ junctions. The downstream primer annealed to sequences 1.4 kb from the 5′ end. (B) R1 5′ junctions. The downstream primer annealed to sequences 2.0 kb from the 5′ end.

for this study as this allowed examination of individual X and Y rDNA loci from each line. For most lines different males exhibited no variation. However, a few of the lines exhibited minor differences in their R1 5′ truncation patterns, which indicated that recent insertions or eliminations were not yet fixed in these lines. All data reported in this study were derived from an individual male of each line.

Figure 2, A and B, shows representative examples of the R2 and R1 5′ junctions obtained by the PCR approach. Each primer combination generated a series of PCR products (bands) that were of the same length in a majority of the Harwich lines, as well as a series of PCR products that were unique to particular lines. Note that while the PCR bands varied in intensity from line to line, the bands within a line were of similar intensity. This uniformity suggested that the elements giving rise to each band were at the same copy number. On the basis of our analysis of the total number of elements in these lines (see below), that level corresponded to one copy per haploid genome. Occasionally fainter bands were detected (*e.g.*, the fainter bands in Figure 2B, line 21). These faint bands were not detected in PCR amplifications using adjacent primers along the element and thus were not scored.

PCR products of the same length observed in all 19 lines were scored as ancestral elements, *i.e.*, present in the original inbred strain that had undergone no eliminations in any line. PCR products missing in 1 or more lines were scored as ancestral elements that had undergone elimination events. In Figure 2A one can observe 4 ancestral R2 truncations; 3 have undergone one elimination and 1 (the 0.59-kb band) has undergone six eliminations. In Figure 2B one can observe 2 ancestral R1 truncations, 1 present in all lines, while the other has undergone six eliminations. In total there were 19 ancestral R1 5′ truncations and 22 ancestral R2 5′ truncations.

PCR bands unique to one line were scored as insertion events. Two examples can be seen in Figure 2A and numerous examples can be seen in Figure 2B. In most cases these new bands were of a length that could be readily distinguished from the bands present in all other lines. However, in a few instances (three cases with R1 and one case with R2), putative new bands comigrated with new bands in a different line. Because we did not observe the elimination of ancestral bands from more than seven lines (see below), we interpreted these few instances of comigrating bands present in two lines as independent insertions rather than the elimination of an ancestral copy from all but two lines. Finally, we also observed instances (two cases with R1 and three cases with R2) in which a band representing an ancestral R1 or R2 truncation became more intense in a line. While these increases could result from either a new insertion whose 5′ truncation was similar to that of an ancestral

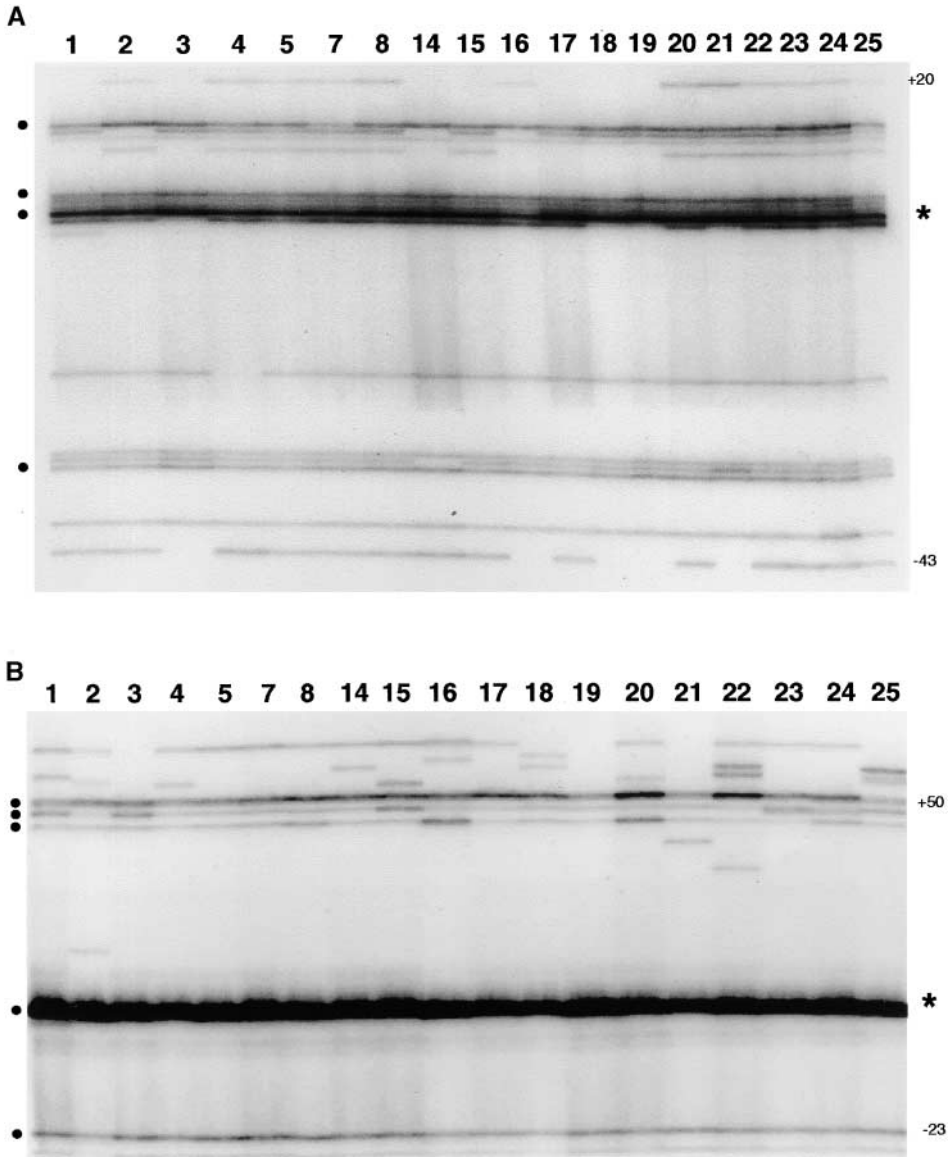

FIGURE 3.—PCR assay to determine sequence variation at the 5′ end of full-length R1 and R2 elements. In both A and B, one PCR primer annealed to the 28S sequence upstream of the insertion site while the second primer annealed to the sequence close to the 5′ end of the element. The primer within the element was end labeled with [γ-^{32}P]dATP. PCR products were separated on high-voltage 8% denaturing polyacrylamide gels. The numbers at the top correspond to the Harwich line number. The asterisks mark the canonical full-length R1 and R2 elements as described by JAKUBCZAK *et al.* (1990). The numbers to the right indicate the size of the band in base pairs relative to this canonical full-length element. Dots to the left indicate bands that correspond to more than one copy per haploid genome. (A) R2 5′ junctions. (B) R1 5′ junctions.

copy or the duplication by recombination of the rDNA unit containing this ancestral copy, they occurred too infrequently to significantly affect our rate calculations and were therefore ignored. In total we observed 197 events involving R1 5′ truncations (163 new insertions and 34 eliminations) and 39 events involving R2 5′ truncations (11 new insertions and 28 eliminations).

Insertion and elimination of full-length R1 and R2 elements: Previous reports have shown that "full-length" R2 elements frequently contain deletions or duplications of the upstream 28S gene sequences as well as short nontemplated insertions (GEORGE *et al.* 1996; BURKE *et al.* 1999; PÉREZ-GONZÁLEZ and EICKBUSH 2001). These variants would also give rise to different-length PCR products, which could be distinguished on high-resolution gels that provide 1-bp resolution. We therefore end labeled the primer that annealed to the sequence 150 bp from the 5′ end of R2 (Figure 1) and after PCR amplification separated the products on high-voltage denaturing polyacrylamide gels. While differing in length, all products generated by this primer set are referred to in this report as full length.

The R2 full-length PCR products are shown in Figure 3A. The canonical full-length R2 element as defined by JAKUBCZAK *et al.* (1990) is the intense band identified with an asterisk. The many bands near this canonical R2 length correspond to full-length copies containing insertions of 1–3 bp of nontemplated sequences or 1- to 2-bp deletions of the 28S gene (GEORGE *et al.* 1996). Other PCR bands in Figure 3A ranged from 20 bp longer to 43 bp shorter than the canonical-length band. The larger bands are likely due to duplications of 28S gene sequences, while the shorter bands correspond to deletions of either 28S or R2 sequences (GEORGE *et al.* 1996; BURKE *et al.* 1999; PÉREZ-GONZÁLEZ and EICKBUSH 2001).

In total 16 bands were scored as present in most or all of the lines and by our definition represent ancestral full-length R2 copies. Four of the bands were of greater

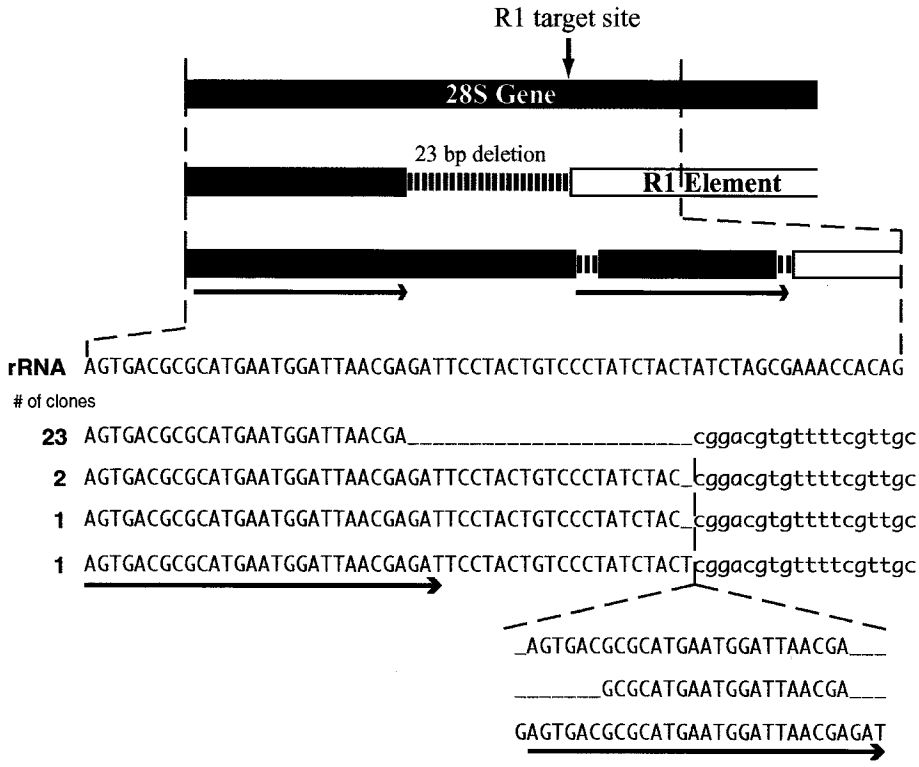

FIGURE 4.—5′ junction sequences of full-length R1 elements. Sequences were derived by PCR from a single male of line 22. At the top are schematics of the 28S gene (solid bars) with or without an R1 insertion (open bars). At the bottom are the sequences of individual cloned junctions. Sequences in uppercase letters correspond to 28S gene sequences, while lowercase letters denote R1 sequences. Of the 27 clones sequenced, 23 were of the canonical full-length R1 elements as defined by JAKUBCZAK *et al.* (1990). This junction contains a 23-bp deletion of the 28S gene (vertical bars). The remaining 4 clones retain these sequences as well as have variable amounts of a 29-bp duplicated 28S gene sequence (denoted by arrows).

intensity, signifying multiple R2 copies with identical-length 5′ ends (bands marked with a dot on the left). We estimated the number of elements represented by these multicopy bands by comparing their intensity to the intensity of the single-copy bands present in the same lanes (see MATERIALS AND METHODS). Each Harwich line contained 15 ± 5 full-length R2 elements with one of these four 5′ junctions. Of the remaining 12 single-copy ancestral full-length R2 variants seen in the PCR analysis (Figure 3A), 6 underwent elimination events in one or more lines (total of 27 events). Only 1 new full-length R2 insertion event is visible in Figure 3A (line 1). Four additional new R2 insertions were identified that gave rise to PCR bands that were 50–150 bp longer than the topmost band shown in Figure 3A. Sequencing revealed that these latter bands corresponded to R2 insertions containing 70–170 bp of what are usually downstream 28S gene sequences now upstream of the R2 element (data not shown). These R2 elements are not inserted into different locations of the 28S gene as PCR analysis using downstream 28S gene sequences in combination with R2 primers indicated that all R2 elements in these lines contained identical 3′ junctions (data not shown). These unusual R2 5′ junctions are therefore the result of large target site duplications generated during the insertion of the element. Elements with large target site duplications may be unstable in the rDNA locus, as none of the ancestral R2 elements in these Harwich lines contained such duplications. Both large and small target site duplications have been previously detected in our survey of R2 junctions in various insect species (BURKE *et al.* 1999).

Unlike R2, sequence variation has not been previously described for full-length R1 junctions (JAKUBCZAK *et al.* 1990; PÉREZ-GONZÁLEZ and EICKBUSH 2001; J. GEORGE, unpublished data). Nevertheless, we also used the end-labeled PCR primer approach to amplify full-length R1 elements with the expectation that occasional full-length R1 variants might be found. The 5′ junction profiles obtained for the full-length R1 elements are shown in Figure 3B. As expected, most R1 junctions were of identical length, giving rise to the intense band indicated by the asterisk. However, similar to the R2 junctions, a small fraction of the full-length R1 elements gave rise to a series of PCR products that were up to 60 bp longer or 26 bp shorter than the predominant junction.

We analyzed the nature of this 5′ variation by cloning and sequencing PCR products from one of the Harwich lines. Most of the clones sequenced (23 out of 32 clones) corresponded to the highly intense PCR band indicated with an asterisk in Figure 3B. This band contained a 23-bp deletion of the 28S gene target site (Figure 4) and corresponded to the R1 junction described in previous reports (ROIHA *et al.* 1981; JAKUBCZAK *et al.* 1990). Four additional clones corresponded to R1 5′ variants larger than this canonical junction. These variants contained a duplication of the 28S sequences typically located upstream of the 23-bp deletion. The presence of the same duplicated sequences in multiple R1 5′ junctions suggests these 28S sequences have retrotransposed along with the R1 element. The five remaining sequenced full-length R1 junctions corresponded to the two shortest-length bands in Figure 3B. These shorter clones con-

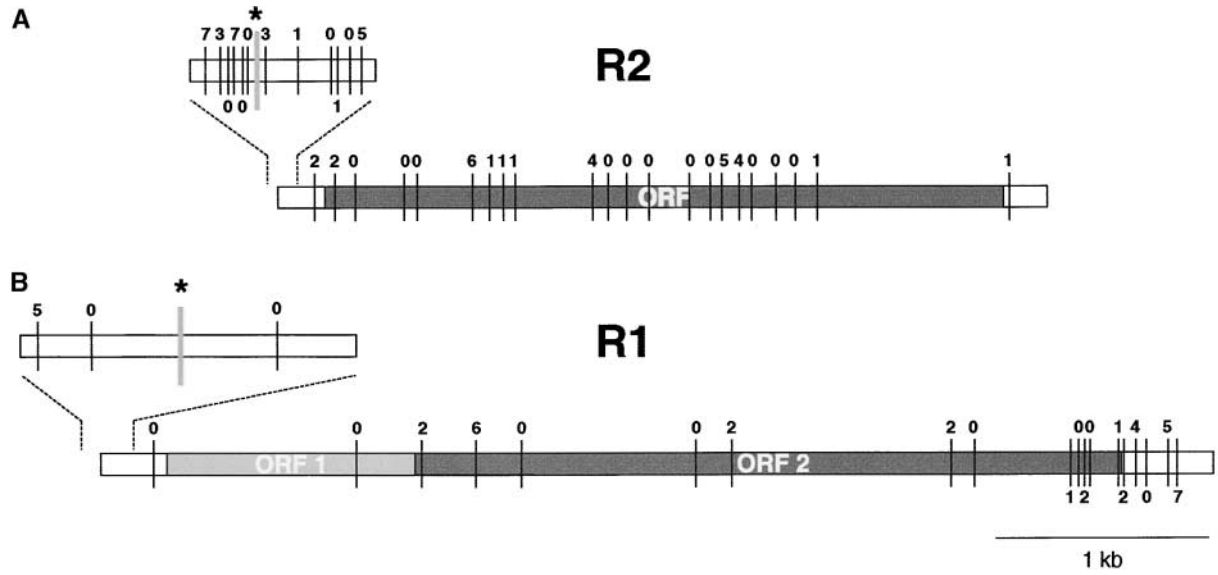

Figure 5.—Summary of the ancestral copies of R1 and R2 elements in the Harwich lines that have unique 5′ sequences. The thin vertical lines denote the length of the 5′ end of these ancestral copies along the length of the R1 and R2 elements. Numbers above or below each line are the number of losses observed for that particular copy among the 19 Harwich lines. The scale at the 5′ end of each element is expanded 10-fold to show the many full-length ancestral copies that differ by only a few base pairs in length. The lengths of the canonical full-length elements as described in Figure 3 are indicated by the thick shaded bar marked with an asterisk.

tained a deletion of an internal segment of the R1 sequence ~80 bp from its 5′ end (sequences not shown). Similar instances in which upstream 28S sequences became part of the retrotransposing element or deletions occurred within the element's 5′ UTR have been seen for R1 and R2 elements in various arthropod species (George *et al.* 1996; Burke *et al.* 1999; George and Eickbush 1999).

Returning to Figure 3B, the ancestral full-length R1 copies were distributed over eight PCR bands. Quantitation of the intensity of the canonical full-length band relative to the single-copy bands in each line (Figure 3B) suggested that the Harwich lines contained from 28 to 62 (average 45) R1 copies with this particular full-length junction. Three of the remaining ancestral bands were of similar intensity to that of the many new insertions detected in the individual lines, indicating that they represented single copies of R1 in each line. Five elimination events were scored, involving these 3 ancestral copies. The copy number of the four remaining ancestral R1 bands varied between 1 and 4 copies per line (bands marked with a dot).

To summarize, our PCR approach permitted the scoring of virtually all insertion and elimination events associated with the 5′ truncated copies of R1 and R2. Unfortunately, only a fraction of the full-length events could be scored because many of these PCR bands were composed of multiple copies. While the intensities of these multicopy bands varied from line to line, clearly signifying turnover, it was not possible to distinguish the number of insertions *vs.* eliminations associated with these differences. Therefore changes in band intensity associated with multiple copies were not included in the analysis below.

Rates and properties of the insertion and elimination events: In total, 34 ancestral R2 elements in the Harwich lines had 5′ junctions, giving rise to unique-length PCR bands (22 5′ truncated and 12 full-length copies). Figure 5A summarizes the location of these unique 5′ junctions (vertical bars) along the R2 element sequence. Based on the relative intensity of the bands in Figure 3A, each Harwich line also contained ~15 ancestral copies of R2 corresponding to full-length insertions with one of four common length variations. In the case of R1, 22 ancestral copies corresponded to unique 5′ junctions (19 5′ truncated and 3 full length; Figure 5B). Approximately 52 additional full-length R1 copies were also present in these lines, most corresponding to one particularly abundant 5′ variant (Figure 3B).

Figure 6 summarizes the 5′ junction positions of the 184 new R1 and 16 new R2 insertions that were detected in all 19 Harwich lines, while Table 2 summarizes the number of new R1 and R2 insertion events scored in each Harwich line. In general, the new R1 and R2 insertions detected in the Harwich lines were characteristic of the ancestral insertions originally present in these lines. For example, while 5′ truncations can occur at almost any position along the length of each element, many of the new R2 insertions were 5′ truncated near the middle of the element. A similar fraction of the ancestral R2 elements is also around this length (compare Figures 5A and 6A). These R2 5′ truncations differ from the R1 5′ truncations (compare Figures 5B and 6B), where a significant fraction of both the new and

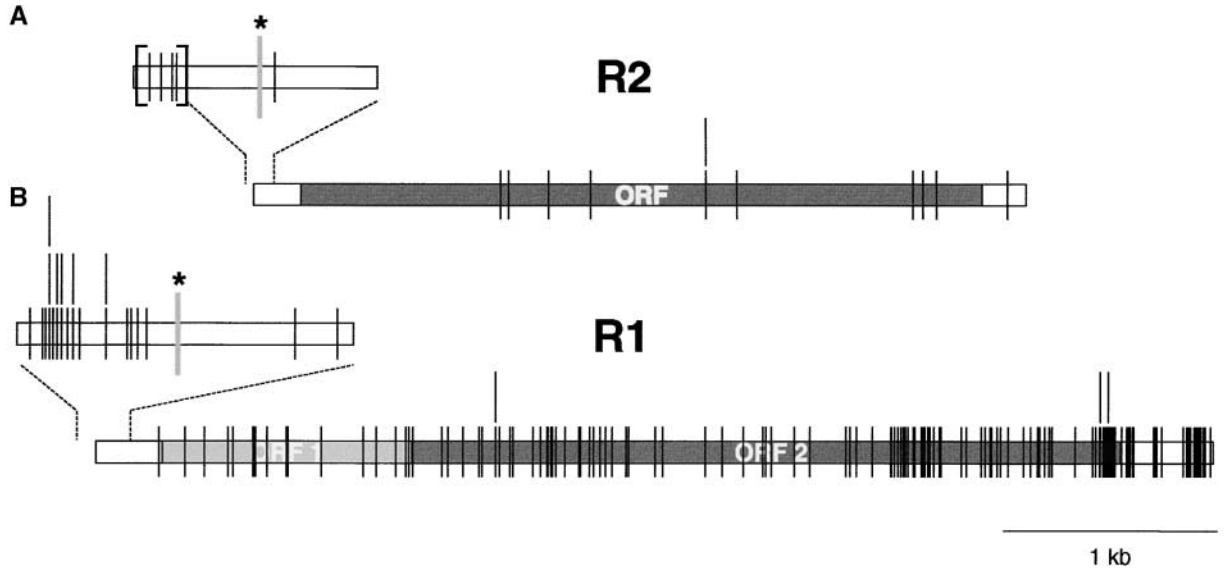

FIGURE 6.—Summary of the new R1 and R2 insertion events scored in all 19 Harwich lines. The thin vertical lines again denote the 5′ ends of the new insertions. New insertions that comigrate in different Harwich lines are denoted by vertical lines placed above another line. The scale at the 5′ end of each element has again been expanded 10-fold. The lengths of canonical full-length elements are again indicated by the thicker shaded bar marked with an asterisk. Vertical bars in the bracketed region at the 5′ end of the R2 element represent full-length R2 insertions with large target site duplications (see text).

old R1 insertions are quite short (*i.e.*, truncated near their 3′ ends).

Because of the many full-length junctions that are of the same length, it is more difficult to determine if the new insertions have the same distribution of full-length to 5′ truncated elements as the ancestral copies. In the case of R1, 22 of the 184 observed new R1 insertions (12.0%) corresponded to full-length elements. This percentage is similar to the fraction of the ancestral R1 insertions that are full-length copies of unique length (3 of 22 or 13.6%). Thus within the limits of our observations, the ratio of full-length to 5′ truncated elements for the new R1 insertions is similar to the ancestral ratio of full-length to 5′ truncated R1 insertions. In the case of R2, only 1 of the 16 new insertions corresponded to a full-length element with a unique 5′ length variant. This contrasts with over one-third of the ancestral full-length R2 elements that corresponded to unique length junctions (12 out of 34). It thus appears that either the fraction of 5′ truncated R2 insertions is increasing in these lines or our experimental approach underestimated the number of new full-length R2 insertions.

If we assume that the fraction of new R1 and R2 insertions observed by our PCR approach (*i.e.*, the appearance of unique 5′ length variants in individual lines) is similar to the fraction of the ancestral copies that have unique 5′ variants, then we scored only ~30% of the R1 insertion events (22 unique 5′ junctions from a total of 74 R1 copies) and nearly 70% of the R2 insertion events (34 unique 5′ junctions from a total of 49 R2 copies). Again the vast majority of the events we missed corresponded to the insertion of full-length copies whose 5′ ends were similar to that of one of the ancestral copies.

The rates of insertion for transposable elements have been estimated by dividing the number of events (gain of a new *in situ* hybridization site on a polytene chromosome) by the number of opportunities (calculated as the number of element copies × number of lines × the number of generations; NUZHDIN and MACKAY 1994; MASIDE *et al.* 2001). We have also used this equation to determine the R1 and R2 retrotransposition rates. We factored into the calculation that the percentage of new elements observed in our PCR approach (*i.e.*, contained a unique 5′ end) was the same as the percentage of ancestral elements that could be scored as unique (30% of the R1 elements and 70% of the R2 elements). By this approach the retrotransposition rate for R1 was calculated to be 12.5×10^{-4} per copy per generation (184 new insertions/22 elements × 19 lines × 353 generations) and for R2 to be 0.7×10^{-4} (16 new insertions/34 elements × 19 × 353).

Within the limits of our resolution these mean retrotransposition rates appear similar for most of the Harwich lines. The number of R1 and R2 insertion events associated with each line is plotted in Figure 7. In the case of R2 (shaded bars), 0–2 new insertions were detected per line (average 0.84 insertions). In the case of R1, many lines had near the average of 9.7 insertions per line (solid bars in Figure 7). However, a few lines had considerably fewer or more numerous insertions, suggesting that the R1 retrotransposition rate may have changed in a few lines.

The number of elimination events associated with

TABLE 2

Numbers of R1 and R2 insertions and eliminations in the Harwich lines

	R1		R2	
Line[a]	Insertions	Eliminations	Insertions	Eliminations
1	8	2	2	5
2	12	1	2	1
3	8	5	1	8
4	9	0	1	4
5	3	2	2	0
7	8	0	0	1
8	7	1	1	0
14	4	1	1	5
15	17	0	0	2
16	9	3	0	4
17	13	1	0	4
18	9	4	1	7
19	5	4	0	6
20	13	2	2	0
21	23	4	0	4
22	10	5	1	1
23	10 (3)[b]	2	1	1 (0)[b]
24	14 (7)[b]	1	1	2 (1)[b]
25	9	1	0	1
Total	184	39	16	55

[a] Numbers correspond to the same subline numbers used in Mackay *et al.* (1992).

[b] Seven R1 insertion events and one R2 elimination were shared between these two lines, suggesting that these lines were cross-contaminated early in their history. Numbers in parentheses are the number of events unique to the respective line. For the total number of events, these shared events were counted only once.

each ancestral R1 or R2 insertion with a unique 5′ junction is shown in Figure 5 (numbers above or below the vertical lines). Thirty-nine eliminations were associated with the 22 ancestral R1 copies, and 55 eliminations were associated with the 34 ancestral R2 copies. The rate of elimination was estimated by again dividing the number of events (loss of an ancestral PCR band) by the number of opportunities (number of ancestral copies × number of lines × the number of generations). Unlike their 18-fold difference in rates of retrotransposition, R1 and R2 exhibited roughly similar rates of elimination: 2.6×10^{-4} eliminations per copy per generation for R1 and 2.3×10^{-4} for R2. Full-length R1 and R2 elements were eliminated at a rate of 3.2×10^{-4} (32 events associated with 15 copies) while 5′ truncated elements were eliminated at a rate of 2.3×10^{-4} (62 events associated with 41 copies). The somewhat faster rate of full-length element elimination is not simply associated with the total length of the insertion because the rate of elimination of the shortest 5′ truncated R1 and R2 copies (insertion lengths <1.0 kb) was 2.8×10^{-4} (21 events for 11 copies), while the rate of elimination of the longest 5′ truncated copies (insertion lengths >2.7 kb) was 2.4×10^{-4} (18 events for 11 copies).

The total numbers of R1 and R2 eliminations per line are summarized in Table 2 and plotted in Figure 8A. While the total number of eliminations averaged 4.9 per line, many lines had only 1 or 2 eliminations, and three lines had at least 10 eliminations. This variance in the number of eliminations per line suggests that either the rate of recombination leading to the elimination of these elements varies between lines or some lines have undergone rare large deletions of their RNA loci, which eliminated multiple R1 and R2 copies in single events.

Finally, the rate of elimination was not the same for all ancestral copies of R1 and R2. Figure 8B shows the number of Harwich lines in which each ancestral copy of R1 or R2 was eliminated (solid bars). Nearly one-half of the ancestral copies (26 of 56 insertions) had no losses in the 19 lines while nine ancestral copies underwent five to seven elimination events. The shaded bars represent the expected number of eliminations per copy if the elimination of each R1 and R2 element occurred at random. The observed data deviate significantly from a simple Poisson distribution (χ^2 test, $P <$

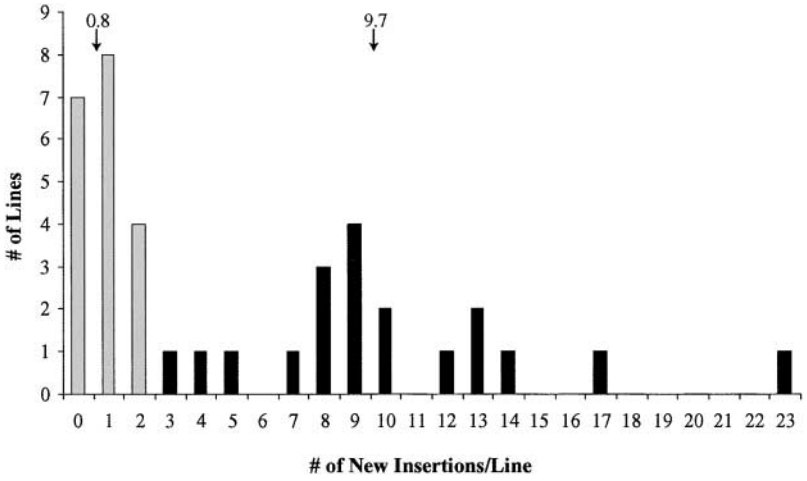

Figure 7.—Distribution of R1 and R2 insertion events in each of the 19 Harwich lines. The shaded bars are the number of new R2 insertions found in each of the 19 lines, while the solid bars represent the number of new R1 insertions found in each line. The average number of events per Harwich line is given above each distribution.

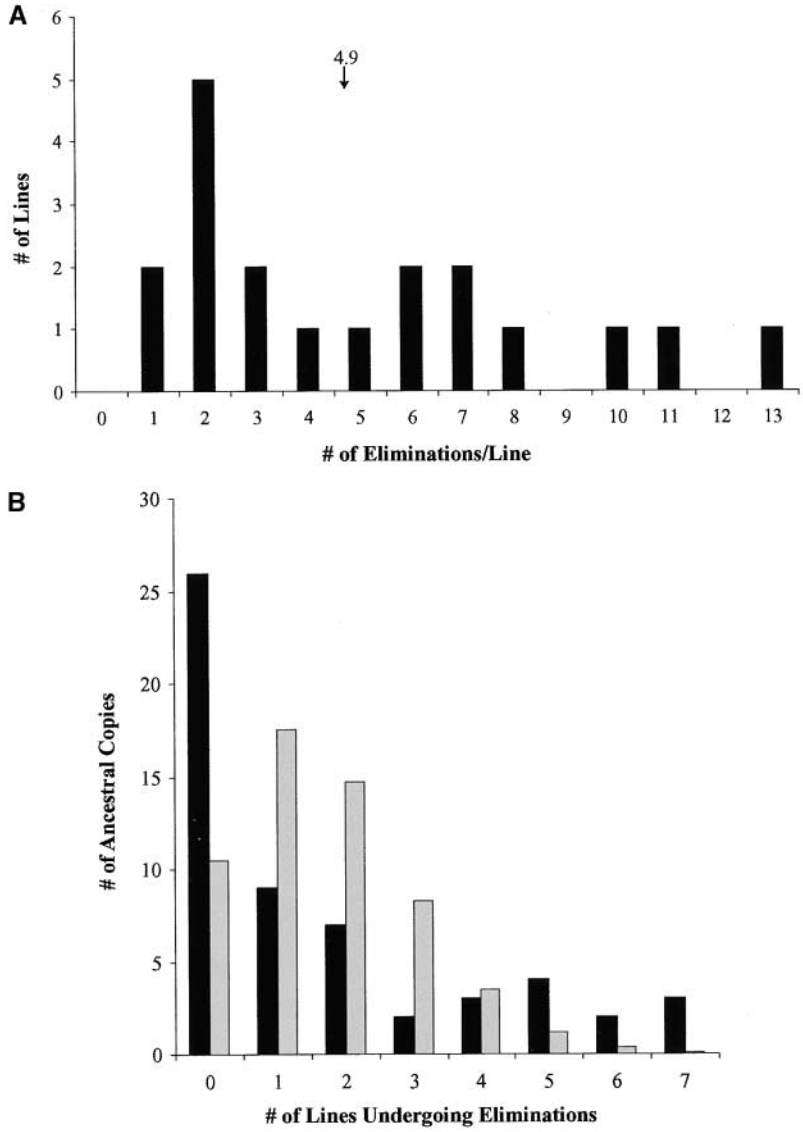

FIGURE 8.—Elimination events in the 19 Harwich lines. The R1 and R2 data have been combined because the rates of elimination of R1 and R2 elements were found to be similar (see text). (A) The number of elimination events in each Harwich line. Solid bars represent the total number of eliminations observed per line (average 4.9 eliminations per line). (B) Frequency of elimination of individual copies of R1 and R2 elements in the Harwich lines. Solid bars correspond to the observed elimination events. The number of times each ancestral copy was eliminated can be seen in Figure 5. Shaded bars are the expected Poisson distribution assuming that R1 and R2 elements are eliminated at random from the array.

0.001), suggesting that all copies are not being eliminated at the same rate. The more rapidly eliminated insertions included both R1 and R2 elements as well as both full-length and 5′ truncated copies (see Figure 5). As is discussed below, one likely explanation for these different rates of elimination is that different regions of the rDNA loci undergo different rates of recombination.

DISCUSSION

Our previous study of a *D. simulans* population revealed that each individual in the population had a unique set of R1 and R2 5′ truncations, a hallmark of active retrotransposition (PÉREZ-GONZÁLEZ and EICKBUSH 2001). Equally important, the vast majority of these 5′ truncations were at one copy per chromosome, indicating that individual insertions were not being expanded by intrachromosomal recombination within the rDNA locus. Intrachromosomal recombination has been shown to be the most rapid form of recombination driving the concerted evolution of the rDNA array (SCHLÖTTERER and TAUTZ 1994). These findings suggested that R1 and R2 elements were not undergoing concerted evolution along with the rRNA genes, but rather the recombinational forces that drive the concerted evolution eliminate these elements from the array. Only by active retrotransposition can R1 and R2 ensure their continued presence in the rDNA locus.

A determination of the absolute rates at which R1 and R2 insertions and eliminations occurred was not possible in the *D. simulans* population survey because we could not trace the appearance and disappearance of individual copies from the rDNA locus. The Harwich lines analyzed in this report were derived from a single highly inbred line and maintained as small populations for a known number of generations. Therefore current differences in the R1 and R2 elements of each line must have arisen since the lines were established. Using 5′ sequence variants generated during the retrotransposition process we could monitor hundreds of events corre-

sponding to the insertion of new copies in individual lines and the elimination of ancestral copies originally present in all lines.

The retrotransposition rates of 12.5×10^{-4} and 0.7×10^{-4}/copy/generation that we determined for R1 and R2, respectively, are similar to the rates determined for other active retrotransposable elements in *D. melanogaster*. Nuzhdin and Mackay (1995) in their survey of these same Harwich lines found no activity for 13 transposable elements, while 4 elements (*Doc*, *copia*, *roo*, and *I*) had rates between 0.4×10^{-4} and 13×10^{-4}/copy/generation. Eggleston *et al.* (1988) surveyed 14 elements and determined that the 2 active elements (*copia* and *roo*) retrotransposed at rates of 2.0×10^{-4} and 0.9×10^{-4}, respectively. In a survey of 4 transposable elements Harada *et al.* (1990) found 2 active elements (*17.6* and *I*) that retrotransposed at rates of 3.8×10^{-4} and 18.0×10^{-4}. Finally, in an analysis of 9 transposable elements Maside *et al.* (2000, 2001) found only 1 active element (*roo*), which retrotransposed at a rate of 4.6×10^{-4} Maside *et al.* (2000, 2001).

While the rates of R1 and R2 retrotransposition are similar to those of other active retrotransposons, the rates we have determined for the elimination of R1 and R2 elements from the rDNA loci, 2.6×10^{-4} and 2.3×10^{-4}, respectively, are much higher than those reported for any other retrotransposon in *D. melanogaster*. Nuzhdin and Mackay (1994, 1995) detected no elimination events for 16 of the transposable elements they examined in these Harwich lines. For the one element, *roo*, in which eliminations were observed, the three events scored gave rise to an elimination rate of 9.0×10^{-6}. Similarly the rare elimination events observed in all other studies of the turnover of retrotransposable elements in *D. melanogaster* suggested elimination rates that are two orders of magnitude below the retrotransposition rates (Eggleston *et al.* 1988; Harada *et al.* 1990; Maside *et al.* 2000, 2001). Interestingly, the eliminations observed in these studies were exclusively associated with LTR retrotransposons and, thus, were likely the result of recombination between the LTRs of the same element rather than complete elimination (Bingham and Zachar 1989; Maside *et al.* 2000). Such extremely low levels of elimination were expected for the retrotransposable elements in these studies because, unlike DNA-mediated elements that excise themselves from one site for insertion elsewhere, the only known means to eliminate a retrotransposable element are ectopic recombination (Charlesworth and Langley 1989) and gradual DNA loss (Petrov *et al.* 1996), mechanisms not expected to be observed in any short-term analysis of mutation accumulation lines. On the other hand, elimination of the non-LTR retrotransposons R1 and R2 from the rDNA locus can be readily explained by the recombinations that result in the concerted evolution of this locus.

The nature of the recombinations within the rDNA locus that give rise to the elimination of R1 and R2 elements is not known. Both unequal crossovers and gene conversions have been postulated to be involved in the concerted evolution of the rDNA loci (Williams *et al.* 1989; Schlötterer and Tautz 1994; Polanco *et al.* 1998). Thus the elimination of R1 and R2 insertions could be the result of gene conversion events removing the insertions from individual rDNA units or the removal of entire rDNA units containing insertions by unequal crossovers between units.

As was first noted in our study of a *D. simulans* population (Pérez-González and Eickbush 2001) most of the 5′ junctions of the truncated R1 and R2 elements in the Harwich lines are present at one copy per genome. In addition, we detected only a few instances (fewer than six) where an ancestral copy appeared to increase in number (*i.e.*, generate a more intense PCR band). Thus the recombinational mechanisms within the rDNA locus seldom duplicate individual copies of R1 and R2. A gene conversion bias against inserted rDNA units is one possible mechanism by which R1 and R2 elements could be preferentially eliminated from the rDNA array. Indeed, gene conversion biases against insertions have been previously noted in other systems (Holliday 1982; Vincent and Petes 1989). On the other hand, if the mechanism removing R1 and R2 copies is unequal crossover, then the bias against inserted units would be more difficult to explain. However, if most intrachromosomal crossover events involving inserted rDNA units are also intramolecular events, then the recombinations would result in the deletion of rDNA units between the sites of recombination (*i.e.*, one product of the recombination would be a circle that would be lost from the cell). To replenish the net loss of rDNA units from the locus with uninserted units would require that the intermolecular unequal crossover events (whether sister chromatid or chromosomal homologs) predominantly involve uninserted rDNA units.

Comparison of the number of elimination events in each of the 19 Harwich lines may provide some clue to the potential recombinational mechanism. Many Harwich lines had only 1 or 2 eliminations while a few lines had >10 eliminations (Figure 8A). It seems unlikely that a gene conversion mechanism individually removing R1 and R2 copies would vary in rate by a factor of 10 between these inbred lines. It seems more likely that the large numbers of eliminations seen in some lines are a consequence of rare large deletions via unequal crossover removing many inserted rDNA units from the array. This unequal crossover model would predict a lower number of rDNA units in those lines with the highest numbers of eliminations, while a gene conversion model would predict similar levels of rDNA units in all lines.

Finally, analysis of R1 and R2 eliminations provides one last clue to the properties of recombination within the rDNA locus. We found that not all copies of R1 and

R2 elements are eliminated at the same rate (Figure 8B). Because these different rates are not associated with the size of the insertion or with the type of element, the simplest model would suggest that these different rates are associated with the different locations of the insertions within the rDNA locus. Prior analyses of tandemly repeated sequences (Hööɢ *et al.* 1988; McAllister and Werren 1999; Schueler *et al.* 2001) have suggested that the least frequently recombining units of an array are near the edges of the array, while the more rapidly recombining units are found at the center of the array. Thus R1 and R2 elements with the highest rates of elimination may be located near the middle of the rDNA loci. We intend to address this issue by physically mapping the R1 and R2 insertions within the rDNA arrays of the Harwich lines. The large numbers of unique R1 and R2 5′ junctions should serve as convenient markers for such an analysis.

Clearly many questions regarding R1 and R2 insertions in these lines need to be addressed. Do R1 and R2 actively insert on both the X and Y chromosomes in males, or do retrotranspositions occur in females and elements obtain access to the Y chromosome only by recombination? Can one estimate the rates and mechanism of recombination within and between the rDNA loci of these lines by monitoring changes in sequence variation associated with the ITS and IGS regions of the rDNA unit (Schlötterer and Tautz 1994; Polanco *et al.* 1998)? Are the insertion and elimination rates constant over many generations or are there periods of high activity over a few generations followed by periods of inactivity? Clearly the Harwich mutation accumulation lines provide an opportunity to address many long-standing questions associated with the concerted evolution of the rDNA loci and the activity of the retrotransposons that occupy these loci.

We thank J. D. Fry, D. G. Eickbush, and W. D. Burke for discussions and comments on this manuscript. We thank S. V. Nuzhdin for providing a subset of the Harwich lines for a preliminary study, T. F. C. Mackay for all the Harwich lines used in this study, W. D. Burke for running the end-labeled PCRs and for help with the figures, H. A. Orr for statistical help, and T. Egborge for help with the R1 analysis. This research was supported by National Science Foundation grant MCB-9974606 to T.H.E.

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Communicating editor: S. Sandmeyer