Chromosome ends in *Drosophila* without telomeric DNA sequences

(chromosome breaks/terminal deletion/telomere/DNA replication/yellow gene)

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ABSTRACT We have recovered terminal chromosome deletions of the X chromosome of *Drosophila* [*Df(1)RT*; *RT* = receding tips] that break in various positions of the yellow gene (*y*) region and delete all distal DNA sequences. Terminal DNA fragments are heterogeneous in length. Molecular cloning and sequencing of the terminal DNA fragments revealed that the broken ends of the deleted chromosomes do not carry any telomeric DNA sequences, yet the broken chromatids do not fuse to one another. Moreover, we confirmed by sequence analysis of 49 independently cloned terminal DNA fragments from two *RT* lines collected at different times that they lose DNA sequences from their distal ends at a rate of 70–75 base pairs per fly generation. We calculate that the rate of loss from these ends is consistent with the removal of an octanucleotide RNA primer at each round of DNA replication in the germ line.

Telomeres perform at least two vital functions. They protect the tip of linear chromosomes from fusion (1, 2), and they allow complete replication of the chromosomal tip (3). The telomeric DNA structure of diverse organisms, including protozoa, fungi, higher plants, and mammals, consists of short, tandemly repeated sequences with one guanosine-rich strand oriented 5' to 3' toward the chromosome end (4–8). The functional conservation of these repeat sequences is reflected in the fact that these sequences from protozoa to humans are recognized as telomeric DNA on the ends of artificial linear minichromosomes in yeast (9–11). Terminal chromosome deletions have been recovered in *Drosophila* that are not subject to fusion (12) but appear not to replicate the end properly. These deleted chromosomes lose DNA sequences from the broken end at a rate of 70–75 base pairs (bp) per fly generation (13). In this paper we demonstrate by molecular cloning and sequencing that the broken chromosome ends of the *RT* (receding tips) deficiencies lack telomeric DNA sequences and address the question of the mechanism for this DNA loss.

MATERIALS AND METHODS

Cloning of Terminal DNA Fragments. Before blunt-end ligation to the mp19 vector, high molecular weight DNA (20 μ g/ml) from adult females was treated with S1 nuclease (100 units/ml) at 37°C for 30 min, followed by a fill-in reaction with *Escherichia coli* DNA polymerase I (large Klenow fragment at 200 units/ml) and all four dNTPs (0.02 mM) for 30 min at room temperature, or was treated with Klenow enzyme and all four dNTPs alone or was used untreated after CsCl gradient centrifugation and phenol/chloroform extraction. DNA (100 μ g/ml) was ligated to the blunt *HincII* site of double-stranded mp19 (also cut with *Pst* I to prevent circularization) at 165 μ g/ml, which represents an $\approx$ 5-fold excess of mp19 blunt ends, by using 740 units of *E. coli* DNA ligase

(Boehringer Mannheim) per ml overnight at 16°C. After ligation, DNA was cut to completion with *Pst* I, diluted to 2 μ g/ml, and circularized at the *Pst* I site by ligation with 36 units of DNA ligase per ml. Each sample of 260 μ l containing 200 ng of DNA was transformed into 1 ml of competent (CaCl_2 method) *E. coli* (strain XL1-Blue; Stratagene) and plated on 150-mm Petri dishes in L broth/0.8% top agarose. The M13 phages, $\approx$ 90% recombinants, were adsorbed to nitrocellulose circles and screened by hybridization with the ^{32}P -nick-translated *y* gene fragment 14b15B in pBR329, terminating at the *EcoRI* site in the second exon of the *y* gene and extending 1.8 kilobases (kb) proximally (13). Sequencing was done by the dideoxynucleotide technique (14).

Collection of Flies, DNA Preparation, and Southern Blot Hybridization. The *y^{RT}* deletions were kept in stock at 18°C as *y^{RT}/y^{2sc}* Y males crossed with *C(1)DX, yf/y^{2sc}* Y females. *y^{RT}/y^{2sc}* Y males were collected and mated with homozygous *Df(1) y ac* virgin females, where *Df(1) y ac* is a small, homozygous viable deficiency that uncovers the *y* and *ac* genes. The *y^{RT}/Df(1) y ac* daughters were collected and frozen at -80°C . DNA preparation and Southern blot hybridization were done as described (13).

BAL-31 Exonuclease Digestion. Genomic DNA was incubated at 30°C at a concentration of 100 μ g/ml in 600 mM NaCl/12 mM CaCl_2 /12 mM MgCl_2 /1 mM EDTA/20 mM Tris-HCl, pH 8.0. After a sample at zero time point was withdrawn, BAL-31 (Promega) was added to 50 units/ml. Aliquots of 40 μ l containing 4 μ g of DNA were taken at 1, 2, 4, and 10 min and pipetted into an equal volume of 20 mM EGTA on ice. After phenol/chloroform extractions, the DNA samples were precipitated with an equal volume of isopropanol, digested to completion with *Bgl* II, and electrophoresed in a 1.1% agarose gel. The Southern blot was hybridized to the nick-translated 14b15B *y* fragment as described above.

RESULTS

We have recovered a number of terminal chromosome deletions that appear genetically and cytologically to have lost the chromosomal tip (12). Although these deletions are equally frequent on all of the chromosomes, we have concentrated on the X chromosome for convenience. Twelve of these deletions [*Df(1)RT*, abbreviated *RT* for receding tips] were selected because they have lost some or all yellow (*y*) gene function, but have not lost the next proximal gene, achaete (*ac*); the approximate positions of their breakpoints within the *y* transcription unit have been determined by Southern blot analysis (13). The *RT* chromosomes are “healed” in that their broken ends do not fuse, suggesting acquisition of some telomeric function, yet they lose DNA sequences from their distal ends at a rate of 50 bp per month, which equals about 70–75 bp per fly generation (13). Similar rates of loss have been described for terminal deletions with breaks at other loci (15–17).

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As we have done previously with other *RT* stocks (13), we determined the position of the chromosome end of *RT627* from flies collected in August 1987 with respect to the *Bgl* II site positioned at the 3' end of the *y* gene (13), and we verified also by its sensitivity to BAL-31 that the heterogeneous band on the genomic Southern blot does indeed represent the terminal DNA fragment of the X chromosome (Fig. 1). The 14b15B hybridization probe from the second exon of the *y* gene hybridized to the same 1.5-kb proximal *Bgl* II fragment in *RT627* and in Oregon RC wild type. In both cases this fragment was insensitive to BAL-31 as expected. In wild-type DNA, the more distal 6.5-kb *Bgl* II fragment, which also hybridized to the 14b15B probe, was also insensitive to BAL-31, confirming its internal position in the wild-type X chromosome. In contrast, the terminal 1.4-kb fragment hybridizing in *RT627* instead of the 6.5-kb fragment in wild-type DNA was shortened rapidly by BAL-31, confirming its terminal location.

To address the question of whether there are telomeric sequences at the distal ends of the *RT* chromosomes, terminal DNA fragments were cloned in the double-stranded M13mp19 vector and screened with a DNA probe from the *y* locus (Fig. 2). Nucleotide sequence analysis was done on 49 independently cloned terminal DNA fragments from two different terminal deficiency stocks (*RT94*, *RT627*) that had been harvested at two different times. The two stocks have a *y*¹ (null) phenotype. The position of the terminal chromosome breaks at the time of harvest, as determined by genomic Southern blots, was located between the proximal end of the

FIG. 1. BAL-31 digestion kinetics of *RT627* DNA (from flies collected in August 1987) and Oregon (ORE) RC wild-type DNA. Genomic DNA was treated for the indicated periods of time with BAL-31, then cleaved with *Bgl* II and electrophoresed in a 1.1% agarose gel. The Southern blot was hybridized to the *y* gene fragment 14b15B terminating at the *Eco*RI site in the second exon of the *y* gene and extending 1.8 kb proximally (13), which hybridizes to a proximal 1.5-kb and a distal 6.5-kb *Bgl* II fragment in wild-type DNA. Both fragments are insensitive to BAL-31, confirming their internal position in the wild-type X chromosome. In *RT627*, the 1.4-kb heterogeneous terminal fragment that hybridizes to the 14b15B probe is shortened drastically by BAL-31, while the next proximal 1.5-kb fragment is not degraded by BAL-31 in the time course of the experiment.

FIG. 2. Cloning protocol of terminal DNA fragments from terminal chromosome deficiencies. For details see *Materials and Methods*.

intron and the distal part of the second exon in the *y* gene (ref. 13; also see below). As expected, all fragments had *y* sequences at their proximal ends, terminating in the *Pst* I site from the second exon of the *y* gene (see ref. 13) that had been used for cloning. The distal ends terminated at various sites within the *y* gene sequence, and none of the fragments carried any additional G+T-rich nucleotide sequences.

Because of the uncertainty of the structure of the end of the *RT* chromosome, DNA was treated in one of three different ways before ligation to the vector: (i) treatment with S1 nuclease followed by a fill-in reaction by DNA polymerase I (Klenow fragment), (ii) treatment with Klenow fragment alone, or (iii) omission of any treatment prior to blunt-end ligation to the mp19 vector (Fig. 2). Such treatments would have made single-stranded 3' and 5' overhangs as well as hairpin loops clonable as blunt ends. All gave inserts of approximately the same length (Table 1), and because yield of terminal fragments recovered per number of screened recombinant M13 phages (ranging from 1/4,000 to 1/14,000) was highest with the untreated DNA, it is reasonable to assume that a large fraction of *RT* chromosomes naturally terminates in a blunt DNA end.

The size range of distal *Pst* I fragments in the *RT94* stock in December 1984 as determined by genomic Southern blot was 1.0 ± 0.3 kb. The average size of the cloned terminal fragments was 861 bp, and the vast majority (85%) of cloned terminal fragments fell within this size range (Table 1 and Fig. 3). Similar results were obtained from the *RT627* stock. In November 1986, the terminal *Pst* I fragments on Southern blots were 1.7 ± 0.3 kb long, and the average size of the cloned terminal fragments was 1513 bp. In August 1987, the terminal *Pst* I fragments were 1.0 ± 0.3 kb, and the average size of the cloned terminal fragments was 861 bp. Thus, within the limits of accuracy of the genomic Southern blots, the size values of the cloned terminal fragments are in good

Table 1. Sizes and breakpoints within the yellow gene of cloned terminal DNA fragments

Clone	Distal end at nucleotide position in yellow gene	Size of insert, bp (Distance from <i>Pst</i> I site)	Clone	Distal end at nucleotide position in yellow gene	Size of insert, bp (Distance from <i>Pst</i> I site)
<i>RT94</i> (January 1984)			<i>RT627</i> (November 1986)		
RT94 N1	2762	1575	RT627 K1	2722	1615
RT94 N2	2587	1750	RT627 K2	2601	1736
RT94 N3	2843	1494	RT627 K3	2856	1481
RT94 N4	3294	1043	RT627 K4	3877	460
RT94 N5	2565	1742	RT627 N1	2482	1855
RT94 N6	3380	957	RT627 N2	2506	1831
<i>RT94</i> (December 1984)			RT627 N3	3318	1019
RT94 SK1	3611	726	RT627 N4	3318	1019
RT94 SK2	3430	907	RT627 N5	2602	1735
RT94 SK3	3361	976	RT627 N6	2532	1805
RT94 SK4	3216	1121	RT627 N7	2677	1660
RT94 SK5	3431	906	RT627 N8	2364	1973
RT94 SK6	3380	957	RT627 N9	3294	1043
RT94 SK7	3380	957	RT627 N10	2386	1951
RT94 SK8	3431	906	<i>RT627</i> (August 1987)		
RT94 K1	3361	976	RT627 K1	3361	976
RT94 K2	3419	918	RT627 K2	3431	906
RT94 K3	3683	654	RT627 K3	3361	967
RT94 K4	3361	976	RT627 N1	3361	976
RT94 N1	3361	976	RT627 N2	3318	1019
RT94 N2	3411	926	RT627 N3	3294	1043
RT94 N3	3260	1077	RT627 N4	3333	1004
RT94 N4	3552	785	RT627 N5	3361	976
RT94 N5	3251	1086	RT627 N6	3431	906
RT94 N6	3895	442	RT627 N7	3380	957
RT94 N7	4234	103			

Individual recombinant mp19 phages harboring terminal DNA fragments from the terminal deficiency stocks *RT94* (harvested in January 1984 and December 1984) and *RT627* (harvested in November 1986 and August 1987) were plaque-purified and sequenced. Column 2 shows the nucleotide position within the *y* gene at which each terminal fragment ends. The *y* gene is transcribed in the distal-to-proximal direction; designation of nucleotides in the *y* transcription unit is according to Geyer *et al.* (18). Column 3 shows the distance of the distal end (same as the size of the cloned DNA insert) in bp from the *Pst* I site in the second exon of *y* gene at nucleotide position 4337. Clones: SK, clones derived from genomic DNA treated with S1 nuclease and Klenow enzyme prior to blunt-end ligation; K, clones derived from genomic DNA treated with Klenow enzyme prior to blunt-end ligation; N, clones derived from untreated genomic DNA. Clone *RT94* SK2 (December 1984) contained an additional 117 bp of non-yellow-gene sequence that will be described elsewhere.

agreement with the measurements from genomic Southern blots. In normal telomeres of yeast, *Tetrahymena*, and trypanosomes, size heterogeneity is likely to be caused by the addition and loss of telomeric repeats (19–24). Even though telomeric repeats are absent from the distal ends of the *RT* deficiencies, the terminal DNA fragments appear diffuse on genomic Southern blots (Fig. 3). Since a restriction enzyme site defines the proximal end of each terminal fragment (*Pst* I in the second exon of the *y* gene), size heterogeneity has to be attributed to the distal end of the fragment, which is also the end of the chromosome. DNA sequence analysis confirmed that the size heterogeneity in the *RT* chromosomes is due to heterogeneity in the position of the distal ends within the *y* gene coding region (Table 1). Some terminal fragments were isolated that terminated at identical nucleotide positions, probably reflecting their higher abundance in the population or a slight sequence preference of RNA primase.

The rate of loss of DNA sequences from the ends of the *RT* chromosomes was determined by sequencing several terminal fragments cloned from the same stock at different times (Table 1). On average, *RT94* appears to have lost 565 bp between January 1984 and December 1984 (51 bp per month), while *RT627* lost 539 bp between November 1986 and August 1987 (60 bp per month), confirming our previous results (13).

DISCUSSION

Telomeres of eukaryotic chromosomes serve a number of functions—e.g., to facilitate the replication of the end of the DNA molecule and to distinguish the natural chromosome

end from a broken end. Mutagen-induced broken chromosome ends are acted upon by DNA repair processes to “fuse” the broken ends together. The result is the restitution of the original chromosome or the production of chromosome rearrangements. Naturally occurring chromosome ends containing telomeres and the *RT* terminal deletions are apparently not subject to this fusion process. Telomeres are also structurally complex, containing tandem copies of G+T-rich repeats as well as other telomere-associated DNA sequences (25) and telomere-specific proteins (26–28). If the ends of the *RT* chromosomes do not have telomere-associated DNA sequences and are constantly losing DNA, the relative stability of these chromosomes cannot be due to the DNA sequences at their ends. A protein that binds specifically to the ends of double-stranded DNA molecules independently of the DNA sequence would be an obvious candidate to protect the ends of chromosomes from DNA repair processes. Such a specific binding property is similar to the characteristics of the Ku protein identified by autoantibodies of some scleroderma patients (29); however, sequence-independent DNA binding of a telomere-specific protein has not yet been demonstrated.

In *Tetrahymena* at least two terminal copies of a telomeric G-T repeat are required for a template-independent addition of more such repeats by a terminal transferase activity (19, 20), and in yeast the telomeric elongation reaction also requires G-T repeats at or close to the end of a linear DNA molecule (21). It is believed that, at normal telomeres, this elongation reaction balances losses due to the inability of the DNA replication machinery to completely replicate the ends

FIG. 3. Size heterogeneity of terminal DNA fragments of *RT94* in December 1984. On the same agarose gel were electrophoresed genomic DNA of *RT94*, cut with *Pst* I, and cloned terminal DNA fragments from the same stock, released from the mp19 vector by *Pst* I/*Bam*HI digest. After Southern transfer, hybridization was done with nick-translated γ gene probe 14b15B. In the genomic DNA lane, the 1.9-kb *Pst* I fragment represents the proximal, internal *Pst* I fragment of the γ gene; the heterogeneous terminal DNA fragments range from 0.7 to 1.3 kb. Sizes of cloned terminal fragments *RT94* SK3, -4, -5, and -6 have been determined by sequencing as 976, 1121, 906, and 957 bp (see Table 1).

of linear chromosomes (3). Eliminating an important component of the enzymatic telomere elongation process by mutagenesis (30), as well as the absence of telomeric repeats at the distal ends of *RT* deficiencies, could prevent terminal transferase action and thus allow progressive loss of nucleotides from such "uncapped" chromosomes.

The *RT* chromosomes are kept in males crossed to attached-X chromosome females. The minimum number of DNA replications per fly generation in the male germ line from fertilized egg to the gonial stem cells has been determined as 18 (31). The stocks are maintained at 18°C and transferred every 6 weeks (13), which is equivalent to a life cycle of 21 days at 25°C. Since embryonic, larval, and pupal development takes about 9 days, this will allow the males to live 12 days as adults. Thus, with a 10-hr cell cycle for gonial stem cells (31), as many as 30 additional rounds of stem cell divisions may occur during the lifetime of a fertile male. No DNA polymerase activity is known that synthesizes in the 3'-to-5' direction. During semiconservative replication, DNA polymerase requires an RNA primer to synthesize the lagging strand. In the absence of a special mechanism to replicate the end of a linear chromosome completely, the chromosomal end is expected to recede at a rate determined by the size of the RNA primer (13), assuming the primase starts at the extreme end of the DNA molecule. Recent evidence (B. Tseng, personal communication) suggests that the primase may, in fact, start at the extreme end of the molecule. Thus in *Drosophila*, which has a primer of eight nucleotides (32), replication is expected to produce one daughter molecule with an eight-nucleotide single-strand overhang and one full-sized daughter. If there is no exonuclease activity, the

next round of replication will yield one daughter of original length, two with an eight-nucleotide overhang, and one with a blunt end shortened by 8 bp. Thus, the average shortening of a linear molecule caused by removal of an octanucleotide primer would be 2 bp per round of replication. With this assumption, we calculate that 36–96 bp will be lost per generation. This agrees well with the observed figure of 70–75 bp per generation.

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