Chromosome end elongation by recombination in the mosquito Anopheles gambiae.

C. W. Roth, F. Kobeski, M. F. Walter and H. Biessmann

One of the functions of telomeres is to counteract the terminal nucleotide loss associated with DNA replication. While the vast majority of eukaryotic organisms maintain their chromosome ends via telomerase, an enzyme system that generates short, tandem repeats on the ends of chromosomes, other mechanisms such as the transposition of retrotransposons or recombination can also be used in some species. Chromosome end regression and extension were studied in a medically important mosquito, the malaria vector *Anopheles gambiae*, to determine how this dipteran insect maintains its chromosome ends. The insertion of a transgenic pUCHsneo plasmid at the left end of chromosome 2 provided a unique marker for measuring the dynamics of the 2L telomere over a period of about 3 years. The terminal length was relatively uniform in the 1993 population with the chromosomes ending within the *white* gene sequence of the inserted transgene. Cloned terminal chromosome fragments did not end in short repeat sequences that could have been synthesized by telomerase. By late 1995, the chromosome ends had become heterogeneous: some had further shortened while other chromosomes had been elongated by regenerating part of the integrated pUCHsneo plasmid. A model is presented for extension of the 2L chromosome by recombination between homologous 2L chromosome ends by using the partial plasmid duplication generated during its original integration. It is postulated that this mechanism is also important in wild-type telomere elongation.

Telomeres are essential chromosomal structures whose functional integrity is linked to cell cycle progression. Considerable efforts are being made to understand telomere structure and to target elongation mechanisms in order to develop new means of proliferation control (12). Every eukaryotic organism must compensate for terminal loss of DNA from chromosome ends because DNA polymerases cannot completely replicate the ends of linear chromosomes. So far, evidence for three different terminal elongation mechanisms has been established. Most eukaryotes have a short, tandemly repeated DNA sequence motif on their chromosome ends. These telomeric tandem arrays are extended by a specific reverse transcriptase, telomerase, which carries an internal RNA template (12). Alternatively, *Drosophila melanogaster* elongates its telomeres by a very different mechanism that is based on transpositions of specific retrotransposons, HeT-A and TART, to chromosome termini (22). In addition, recombination of repetitive telomeric ends has been considered as a possible elongation mechanism (4, 10, 42). This pathway has been well documented in yeast where telomeres are extended by telomerase, but recombination can be used as an efficient bypass mechanism for elongation (19, 20, 30, 39).

Understanding the mechanism of telomere elongation in the malaria-transmitting mosquito *Anopheles gambiae* may be useful for developing new strategies for vector control. Therefore, we examined the dynamics of chromosome length variation at the 2L chromosome end. For our studies, we used strain G418 of *A. gambiae* that resulted from attempts to transform *A. gambiae* by injecting the *D. melanogaster* P element construct pUCHsneo into mosquito embryos (25). The plasmid conferring resistance to the neomycin analog G418 to the transformed mosquitoes integrated fortuitously into the extreme end of chromosome 2L (11). We have recently reported on the cloning and characterization of the *A. gambiae* flanking DNA at the proximal end of the integration, which consists of a tandem array of 0.8-kb repeats (3). In the present study, we cloned and sequenced the 2L chromosome end and examined the dynamics of chromosome length variation. We demonstrated telomere shortening as well as elongation. The extension of the newly elongated chromosomes is an exact copy of the original transgene sequence including the generation of a new pUC8 polylinker. We propose that the extensions are created by unequal crossing over between homologous 2L ends containing a tandem integration of a complete and a partial copy of the transgene.

**Materials and Methods**

**Mosquito Maintenance.** The G418 mosquito stock was derived from the G3 strain of *A. gambiae* by transformation with pUCHsneo (25). It is homozygous for the transgene. The transformed stock has been grown continuously since its isolation in 1986 under G418 selection (400 mg/ml) by the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. In July 1996, the G418 stock was split, and one half was maintained with G418 selection and the other half was raised without G418 selection. With the growth conditions employed the generation time is about 3 weeks.

**Genomic DNA isolation.** To isolate genomic DNA from single mosquitoes or from pools of 50, the mosquitoes were homogenized on ice in 100 μl of lysis buffer containing 100 mM potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4). DNA from pools of mosquitoes was filtered through glass wool into 1-ml aliquots. The solutions were spun at 4°C for 2 min, and the supernatants were discarded. Pellets were taken up in 100 μl (500 μl for pools) of lysis buffer containing 10% sodium chloride and 15% sodium potassium chloride, 1.5 mM spermine, 0.5 mM spermidine, and 15 mM Tris- HCl (pH 7.4).
tate was added to 0.1 M, and DNA was precipitated with an equal volume of isopropanol and centrifuged for 5 min. DNA was carefully dissolved in 50 μl (400 μl for pools) of TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA), and after addition of sodium acetate, it was precipitated with 2.5 volumes of ice-cold ethanol. After centrifugation, DNA was dissolved in 10 μl (50 μl for pools) of TE.

Southern blotting and hybridizations. Genomic DNA was digested with a 10-fold excess of restriction enzymes (Gibco/BRL) in the presence of 5 mM spermidine for 3 h. Three milligrams of genomic DNA from the pooled DNA (or one half) isolated from single mosquitoes was electrophoresed in 1% agarose gels in Tris-acetate buffer. After denaturation in the gel, DNA was transferred for 36 h by capillary action in 20X SSC (3 M NaCl–1.5 M sodium citrate) and 0.2% Hybond N+ nylon sheets (Amersham). DNA was cross-linked by UV (Stratalinker; Stratagene), preincubated for 1 h in 0.1× SSC–0.5% sodium dodecyl sulfate (SDS) at 65°C, and then incubated for 5 h in hybridization buffer at 42°C. Hybridization was done at 42°C for 36 h with [32P]dCTP (NEN) random-primer (Stratagene) DNA probes in a solution containing 50× denatized formaldehyde, 5× SSC, 50 mM Tris-HCl (pH 7.6), 10× Denhardt’s solution (0.2% [each] Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 0.1% SDS, 2.5 mM EDTA, and 0.1 mg/ml sheared and denatured salmon sperm DNA. Filters were washed at 80°C in 0.1× SSC–0.1% SDS for 2 h, dried, and exposed on X-Omat AR film (Kodak) with Quanta III intensifying screens (DuPont) at −70°C. As a hybridization probe we used the 1,556-bp fragment between the XhoI site in neo and the HindIII site (see Fig. 1) in the P element sequences of the pUCChsneo transgene (36). The fragment was purified by gel electrophoresis in low-melting-point agarose, and 20 to 50 ng was used for labeling by a random-primer reaction (Prime-it II; Stratagene).

Terminal cloning by plasmid rescue. The terminal sequences of chromosome 2L were cloned from 1 to 5 μg of high-molecular-weight DNA. The DNA was treated with either T4 polymerase (Boehringer) or with mung bean nuclease (Gibco/BRL) to create blunt ends. T4 polymerase (3 U) was incubated with the DNA in the presence of 0.25 mM (each) deoxynucleotide for 30 min at 37°C and then inactivated at 60°C for 10 min. DNA was incubated with 56 U of mung bean nuclease at 30°C for 30 min. The DNA was then treated with phenol–chloroform (1:1), precipitated, resuspended in water, and cut with the appropriate restriction enzyme, Smal or HindIII, in the buffer supplied plus 5 mM spermidine. After the DNA was digested, it was heated to 65°C to inactivate the restriction enzyme, treated with phenol–chloroform (1:1), precipitated, resuspended in water, and brought to 0.5 ml of 1× ligation buffer without polyethylene glycol. Fifty units of T4 DNA ligase (10 U/μl, Gibco/BRL) were added, and the nucleotide reaction was incubated for 12 to 18 h at 20°C. The DNA was concentrated, treated with phenol–chloroform (1:1), and precipitated. The DNA was finally dissolved in 20 μl of water. The ligated DNA was introduced into Escherichia coli DH5α cells with a Bio-Rad electroporation apparatus, and transformed cells were selected on plates containing 100 μg of ampicillin/ml.

DNA amplification by PCR. PCR with genomic DNA were done with the long-range PCR amplification kit from TaKaRa Shuzo Co., Otu Shiga, Japan. Reaction mixtures (50 μl) contained 40 to 100 ng of genomic DNA, 400 μM (each) deoxynucleoside triphosphate, 1× LA PCR buffer (supplied by the manufacturer), 16 ng of each primer, and 2.5 U of Ex Taq polymerase (TaKaRa). Primers obtained from Genosin Biotechnologies, Inc., were used in various pairwise combinations. Their nucleotide positions on the pUCChsneo construct, as numbered in reference 36, are given in parentheses, and their locations on the pUCChsneo transgene in the G418 stock are shown in Fig. 7A. The primers used were neoF1, 5′-CCGCAACATGTCGACCTGAGGAG3′ (3171 to 3190); neoF2, 5′-CATGGTCGAGGACCTGTCGTC3′ (3533 to 3553); neoF3, 5′-GCCTCTTACATGAAAGTTGG3′ (4579 to 4598); and pUCRI, 5′-GCTCACATGTCGACCTTCTGCG3′ (1100 to 1080) and pUCR2, 5′-CCACCTCTGTCGACCTGTCG3′ (1212 to 1192). PCRs were done in a Crocodile III thermocycler (Appligene Oncor, Inc.) at an annealing temperature of 65°C, with 3 min of synthesis at 74°C and a total of 25 cycles. A total of 3 to 10 μl of each reaction mixture was loaded on a 1% agarose gel for analysis of amplified DNA products.

DNA sequencing. Sequencing was done from double-stranded plasmids by using Sequenase 2.0 according to the manual of the United States Biochemical Co. To sequence across the pUC8 polylinker in the plasmid-rescued constructs, the M13 reverse primer was used. Sequence alignments were made with the known sequence of pUCChsneo (36). Sequence analyses were done with the DNA Strider program (21).

**RESULTS**

2L chromosome ends in 1993. In G418-resistant mosquitoes collected in August 1993, the 2L chromosome end was mapped by Southern blotting and showings near the distal end of the integrated pUCChsneo transgene. Due to size heterogeneity, the terminal DNA fragments from a pool of approximately 50 mosquitoes appeared as a broad band characteristic of chromosome termini (3). In order to assess the extent of size polymorphism at the telomere, DNA from individual mosquitoes was assayed. BamHI and PstI cut the minisatellite immediately proximal to the pUCChsneo insert as well as at known positions within it (Fig. 1). This technique allowed us to determine the length of terminal fragments from two different reference points by genomic Southern blot analysis (Fig. 1A). Since the BamHI site is located 3.1 kb proximal to the distal PstI site in the pUCChsneo insert (Fig. 1B), the lengths of the corresponding BamHI- and PstI-generated fragments from each individual mosquito are consistent with their terminal location. While internal bands are identical in all individuals, the terminal fragments vary from individual to individual within a narrow range of about 200 bp (Fig. 1A). Assuming that the pUCChsneo sequences are not rearranged distally to the neo gene, these measurements would position the ends of these 2L chromosomes within the *Drosophila* white gene. The same technique was then used to assess the positions of 50 chromosome 2L ends by assaying 10 pools of five mosquitoes each (data not shown) to determine if there were any longer terminal fragments in the population. No ends significantly
The finding that each clone ends in the cloning procedure, it is unlikely that a large sequence was lost. Although a few nucleotides may have been lost during the fragments cloned represent the true ends of chromosome 2L. The cloned points of termination are in good agreement with the predicted endpoints based on the distance from the neo gene shown in Fig. 1 and 4. The apparent range of chromosome 2L ends 1993 DNA; open box, the last identical nucleotide in the 1995 DNA which were followed by a C residue of the half-SmaI or HincII site used for circularization.

FIG. 2. The positions of chromosome 2L termini in mosquitoes harvested in 1993, 1995, and 1996. The sequence from the beginning of the coding region of the neo gene through the white gene is numbered according to reference 36. The important features of the sequence are written above the sequence. The boxed PstI site is the distal-most PstI site in the neo gene shown in Fig. 1 and 4. The apparent range of chromosome 2L ends 1993 DNA; open box, the last identical nucleotide in the 1995 DNA which was followed by a C residue of the half-SmaI or HincII site used for circularization.

FIG. 3. Autoradiograph of genomic Southern blots of 1995 mosquito DNA hybridized to the neo probe. Genomic DNA from 10 G418 mosquitoes (indicated by numbers at the top of the gel) collected in December 1995 was divided in half and digested with BamHI or PstI, respectively. The internal BamHI (2.9 kb) and PstI (2.45 kb) fragments are the same as those in August 1993 (see Fig. 1). The other bands reflect terminal DNA fragments representing five different 2L chromosome ends termed A to E (Fig. 4). Chromosome A is about 1 kb longer than the average 2L chromosome in August 1993, and chromosome B is about the same length as in the 1995 samples. It appears that these two chromosome groups have undergone terminal elongation since 1993. The shorter chromosomes, C, D, and E, may be considered descendants of the 2L chromosomes sampled in 1993 which have suffered terminal shortening. By averaging their sizes with respect to the PstI site and subtracting from the average size of the terminal fragments in August 1993, we estimate that the 2L tip has receded about 1.0 kb in 28 months. With a generation time of about 3 weeks, a terminal loss of 27 bp/mosquito generation has been calculated. Possible reasons are discussed below.

The positions of 2L chromosome termini in August 1993 and in December 1995 are depicted in Fig. 4. All chromosomes in August 1993 extended past the distal neo gene, but only chromosomes A and B in December 1995 are long enough to contain a functional neo gene. The short chromosomes C, D, and E appear to terminate within the neo gene and, therefore, should not confer resistance to G418. Chromosome B terminates within the Drosophila white gene, and chromosome A terminates about 600 bp beyond the presumed end of the white gene. The other mosquitoes carry heterozygous 2L chromosome ends as follows: mosquitoes 1 and 2, 1 and 3, 1 and 4; and 2 and 3, 4 and 5, and 6 and 7. Since the distal-most PstI fragment in the transgene is positioned 3.1 kb distal of the neo gene, but only chromosome A and mosquito 10 appears homologous for chromosome B. The other mosquitoes carry heterozygous 2L chromosome ends as follows: mosquitoes 1 and 2, 3 and 4; and 5 and 6. Since the distal-most PstI fragment in the transgene is positioned 3.1 kb distal of the neo gene, but only chromosome A and mosquito 10 appears homologous for chromosome B. The other mosquitoes carry heterozygous 2L chromosome ends as follows: mosquitoes 1 and 2, 3 and 4; and 5 and 6. Since the distal-most PstI fragment in the transgene is positioned 3.1 kb distal of the neo gene, but only chromosome A and mosquito 10 appears homologous for chromosome B. The other mosquitoes carry heterozygous 2L chromosome ends as follows: mosquitoes 1 and 2, 3 and 4; and 5 and 6. Since the distal-most PstI fragment in the transgene is positioned 3.1 kb distal of the neo gene, but only chromosome A and mosquito 10 appears homologous for chromosome B. The other mosquitoes carry heterozygous 2L chromosome ends as follows: mosquitoes 1 and 2, 3 and 4; and 5 and 6. Since the distal-most PstI fragment in the transgene is positioned 3.1 kb distal of the neo gene, but only chromosome A and mosquito 10 appears homologous for chromosome B.
gene sequences. Every individual analyzed in December 1995 contains at least one of the two longer chromosome classes, and thus no G418-resistant mosquitoes were found with only the short chromosomes C, D, or E. Of 20 mosquitoes analyzed, 17 were heterozygous for 2L, two were homozygous for chromosome A, and one was homozygous for B. The frequencies of 2L chromosomes in the population were as follows: A, 11 of 40 (27.5%); B, 14 of 40 (35%); C, 5 of 40 (12.5%); D, 8 of 40 (20%); and E, 2 of 40 (5%).

To further study the dynamics of telomere growth in this strain, it was again sampled in June 1996. DNA prepared from a pool of 50 mosquitoes was cut with several restriction enzymes, and the Southern blot was hybridized to the neo gene probe (Fig. 5A). The map of the 2L chromosome ends present in the population in June 1996 is shown in Fig. 5B. With enzymes that cut the pUChsneo transgene only once in the pUC8 polylinker (SalI, EcoRI, and BamHI), the same abundant 5.4-kb fragment hybridizes to the neo probe. This fragment is identical in size to the terminal fragment of chromosome A present in lower abundance in the December 1995 population (27.5%, see above). This fragment was not detected in the 1993 population (see Fig. 1) and must have arisen in the population between August 1993 and December 1995. As we will demonstrate below, this common fragment originates from the presence of a second pUC8 polylinker positioned 5.4 kb (about 1 unit length of the pUChsneo transgene) distal to the polylinker in the original transgene. The >10-kb bands represent proximal fragments. The strong bands at 3.4 kb (XhoI) and 2.9 kb (EagI) extend from the corresponding single sites for these enzymes in the transgene to the end of the chromosome, which would position the physical end of this 2L chromosome 0.5 kb beyond the newly acquired, distal pUC8 polylinker. This conclusion is supported by the fact that the EagI fragment is 0.5 kb shorter than the XhoI fragment, reflecting the distance between the two sites in the transgene and arguing for superposition of ulation. Their sizes as measured from the pUC8 polylinker (4.5, 3.7, and 3.5 kb, respectively) are consistent with the positions of chromosome ends B, C, and D determined in December 1995 (see Fig. 3 and 4). With DNA preparations from single mosquitoes it was shown that these terminal length polymorphisms represented distinct chromosomes which could be segregated. Our interpretations are supported by digesting the genomic DNA with XhoI and EagI, which do not cut in the pUC8 polylinker. The >10-kb bands represent proximal fragments. The strong bands at 3.4 kb (XhoI) and 2.9 kb (EagI) extend from the corresponding single sites for these enzymes in the transgene to the end of the chromosome, which would position the physical end of this 2L chromosome 0.5 kb beyond the newly acquired, distal pUC8 polylinker. This conclusion is supported by the fact that the EagI fragment is 0.5 kb shorter than the XhoI fragment, reflecting the distance between the two sites in the transgene and arguing for superposition of
FIG. 6. Autoradiograph of a genomic Southern blot demonstrating terminal nucleotide loss from the 2L chromosome end. DNA was prepared from pools of 50 mosquitoes of six consecutive generations of the G418 strain after the selection with G418 was discontinued in July 1996; cut with BamHI (B), XhoI (X), and EagI (E); and hybridized with the neo probe. As in Fig. 5, the >10-kb bands represent proximal fragments, and the 5.4-kb BamHI band represents the fragment between the two pUC8 polylinkers (see Fig. 5B and 7A). While the internal BamHI band at 2.9 kb (see map in Fig. 1) remains unchanged, the terminal 3.4-kb XhoI and the 2.9-kb EagI bands (see map in Fig. 5B) become gradually shorter and more heterogeneous in size, indicating their position at the end of the 2L chromosome.

The terminal 3.4-kb XhoI band at 2.9 kb (see map in Fig. 1) remains unchanged, the terminal 3.4-kb XhoI and the 2.9-kb EagI bands (see map in Fig. 5B) become gradually shorter and more heterogeneous in size, indicating their position at the end of the 2L chromosome. The terminal 3.4-kb XhoI band at 2.9 kb (see map in Fig. 1) remains unchanged, the terminal 3.4-kb XhoI and the 2.9-kb EagI bands (see map in Fig. 5B) become gradually shorter and more heterogeneous in size, indicating their position at the end of the 2L chromosome.
collection (lane 6). Similarly, the primer combination neoF1-pUCR2 yielded the expected 100-bp-larger fragment from the 1996 collection but again did not amplify any fragment from the 1993 collection. These results clearly demonstrate that the distal-most extension in 1996 consists of pUC8 and extends at least 450 bp distal to the new pUC8 polylinker.

As positive controls, the primer combinations neoF2-pUCR1 (lanes 3 and 8), neoF2-pUCR2 (lanes 4 and 9), and neoF3-pUCR1 (lanes 5 and 10) amplified the expected 2.8-, 2.9-, and 2.2-kb fragments, respectively, from both mosquito collections. These primer combinations would occur twice in the DNA of the 1996 stock, but the identical sizes of amplified fragments from 1993 and 1996 suggest that the distal portion of the transgene in 1996 is a faithful copy of the proximal sequences. The amplified fragments hybridized to the neo probe and contained the correct restriction sites (data not shown), confirming their predicted positions on the map in Fig. 7A.

**DISCUSSION**

Telomere dynamics. The dynamics of end regression and expansion at the 2L chromosome arm have been studied in a transgenic strain of *A. gambiae* over a period of about 3 years. Our results reveal some dramatic effects and provide evidence for telomere shortening as well as elongation. Mosquitoes harvested in 1993 were fairly synchronized with regard to the length of chromosome 2L, and all 2L chromosome ends were within a range of about 500 bp. Conversely, the population in 1995 contained chromosomes of significantly different terminal lengths. Some of the 2L ends were shorter than those in 1993 mosquitoies, consistent with end regression in the absence of continuous elongation. Using the difference in chromosome length between the shorter 1995 chromosome ends and those in 1993, together with the terminal shortening determined directly in the 1996 population, we estimate that the 2L chromosome shortens at a rate of about 27 to 40 bp per generation.

This terminal regression is slower than in *D. melanogaster*, where the telomeres shorten at 75 bp/generation (1, 2, 5, 17). Terminal shortening is caused by the inability to completely replicate the ends of linear DNA molecules and possibly also by other factors such as nuclease activity. While the effects of various parameters are not known in *A. gambiae*, it appears likely that the slower shortening is due to fewer germ line cell divisions in *A. gambiae* than in *Drosophila*. Comparing terminal shortening rates calculated from Fig. 4 and 6, there does not seem to be a dramatic difference between shortening in the presence or absence of G418 selection.

The 1995-1996 population had a large number of members with chromosomes as long as or longer than those observed in 1993, indicating that there had also been chromosome elongation events in the population. The longer chromosomes may have been present in the 1993 population at a very low abundance and may have been selected for by the continuous G418 selection requiring a functional neo gene. The longest chromosomes were found to be about 1.6 kb longer than those measured in 1993. Southern blotting, PCR analyses, and sequence data indicate that the elongated DNA is derived from the transgene pUChsneo sequence. This fact is also demonstrated by the recovery of normal full-length copies of the pUChsneo plasmid from the longest chromosomes by using DNA restricted with enzymes that cut in the pUC8 polylinker but not in the other transgene sequences. Digestion with *Xho*I or *Eag*I, which cut the transgene once in the neo gene, indicate that the chromosome extends approximately 500 bp beyond the distal polylinker, and PCR data showed that the newly added sequences are a continuation of the pUC8 sequence. Chromosomes that are the same size as those observed in 1993 must have also been elongated, since the 1993 chromosomes should have lost about 1,200 bp in the intervening 2.5 years. Like those in 1993, they end in the white gene of the pUChsneo sequence, and like all the sequenced termini, the sequenced member of this class ends without any repeat sequence.
Model for 2L elongation. The simplest model to explain the observations that the 2L chromosome extensions are composed of sequences already present near the end of the chromosome and do not contain any new sequences typical of telomerase elongation or transpositions is that the chromosomes elongate by an unequal crossing over mechanism. The early mapping of the pUChsneo transgene in the G418 A. gambiae stock (11) together with recent detailed studies (3) indicated that a complete and a partial copy of the transgene were integrated at or near the end of the chromosome. We propose that this chance integration allowed elongation of the chromosome by terminal gene conversion as shown diagrammatically in Fig. 8. Mechanisms of regenerating chromosome ends via recombination have been discussed previously (4, 10, 42), and although telomerase-mediated elongation is the major elongation pathway in most eukaryotes (13), alternative mechanisms using recombination exist in yeasts (19, 39) and possibly in some human cell lines (8, 28, 32, 37). When telomere elongation by telomerase is inhibited in some human cell lines (8, 28, 32, 37), telomeres become gradually shorter and the cells senesce and die. However, recombination as a way to extend chromosome ends is observed in the survivors (20).

Our study shows that the original pUChsneo transgene can be used for terminal elongation. Does the end maintenance discovered in this study reflect wild-type telomere behavior? We cannot answer this question definitively until we have cloned the terminal fragment of a wild-type telomere, but our results allow some reasonable deductions. In most organisms broken chromosome ends are healed by the same mechanism that is also used for wild-type telomere elongation. Organisms that elongate their telomeres by telomerase usually heal broken chromosome ends by the addition of telomeric repeats, which are most likely generated by telomerase (15, 27, 33, 40, 41) although yeast may use either telomerase or recombination (14). Broken chromosome ends in Drosophila are extended by the addition of retrotransposable elements which also maintain normal chromosome ends (2, 6, 7, 34, 38). We detected no sequences resembling telomerase-generated short repeats at the end of chromosome 2L in stock G418, arguing against an involvement of telomerase in the observed elongation event.

Could such repeats have been present at the end of chromosome 2L before the addition of the transgene sequences? There is no evidence for the presence of typical telomere repeat sequences in the genome of any dipteran insects studied to date by cross-hybridization with repeats from other eukaryotes (3, 16, 24, 29, 31, 43). Instead, telomeres from dipteran insects studied to date are very different. Drosophila chromosomes terminate in a series of variable-length HeT-A and TART retroposon arrays, while Chironomus chromosomes terminate in long blocks of 350-bp repeats (18). For these reasons, we predict that wild-type Anopheles chromosomes will also be devoid of typical telomere repeat sequences. We also found no evidence for elongation of the 2L telomere by transposable elements, similar to the situation in Drosophila, where chromosomes are extended by the addition of retroposons (22). In the G418 mosquito stock no additional new sequence different from the pUChsneo sequence was observed at the end of chromosome 2L. Although more chromosome termini need to be studied over a longer period of time to detect possible rare elongation events by transposition, our preliminary observation argues that mosquitoes do not use retroposons to extend either their broken or their normal chromosome ends as Drosophila does.

In summary, we find negative evidence for the telomerase and transposable element elongation mechanisms but positive evidence for elongation by recombination. We propose that the experimental situation at the 2L chromosome end studied here resembles the elongation of normal telomere ends in A. gambiae. The normal mosquito telomeres may be made of tandemly repeated sequences such as the minisatellite discovered earlier (3), and elongation by recombination between multiple repeats would be much more efficient than that between the complete and partial copies of the transgene at 2L. In order to prevent these chromosome ends from being detected by cell cycle checkpoints as double-strand breaks, they may be protected by a terminal binding protein without sequence specificity, similar to the human protein Ku (26), as we have proposed earlier for Drosophila telomeres (1). A telomere organization in A. gambiae consisting of a complex satellite structure which extends to the chromosome end would be comparable to the organization at the telomeres of Chironomus sp., a related dipteran insect. It has recently been proposed that Chironomus may regenerate its telomere ends by a recombination mechanism involving long, complex terminal repeats (9, 18). The observed unusual telomere elongation strategies in dipteran insects suggest that these organisms with relatively large genetic content in relatively small numbers of chromosomes may have different selective pressures determining the mechanism regulating chromosome end maintenance.

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FIG. 8. Model of terminal elongation by gene conversion involving the pUChsneo transgene at the 2L telomere of A. gambiae.
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