MOLECULAR AND CELLULAR BIOLOGY, Jan. 1988, p. 251–258
0270-7306/88/010251-08$02.00/0

Vol. 8, No. 1

Developmentally Controlled Telomere Addition in Wild-Type and Mutant Paramecia

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Received 11 June 1987/Accepted 19 October 1987

We analyzed sites of macronuclear telomere addition at a single genetic locus in *Paramecium tetraurelia*. We showed that in homozygous wild-type cells, differential genomic processing during macronuclear development resulted in the A surface antigen gene being located 8, 13, or 26 kilobases upstream from a macronuclear telomere. We describe variable rearrangements that occurred at the telomere 8 kilobases from the A gene. A mutant (d48) that forms a telomere near the 5′ end of the A gene was also analyzed. This mutant was shown to create simple terminal deletions; telomeric repeats were added directly to the truncated wild-type A gene sequence. In both the mutant and wild-type cells, the telomeric sequences (a mixture of C4A2 and C3A3 repeats) were added to various sequences within a specific 200- to 500-base-pair region rather than to a single site. No similarities were found in the primary sequences surrounding the telomere addition sites. The mutation in d48 changed the region of telomere addition at the A gene locus; this is the first example in ciliates of a mutation that affects the site of telomere addition.

Developmentally controlled formation of new telomeres occurs in diverse eucaryotes. There is cytological evidence for de novo telomere formation in *Ascaris* and the crustacean *Cyclops* (reviewed in reference 29) and molecular evidence for its occurrence in ciliated protozoans. Ciliates contain two functionally and morphologically distinct types of nuclei within a single cell. Both types, micronuclei and macronuclei, arise from a single diploid fertilization nucleus during a sexual event. In the ciliate *Paramecium tetraurelia*, the micronuclei remain diploid and are transcriptionally inactive, while the DNA in the transcriptionally active macronucleus is amplified to about 800C. The formation of the new macronucleus involves the loss and rearrangement of sequences from the germ line DNA as well as chromosomal fragmentation (reviewed in reference 5). Fragmentation of the micronuclear chromosomes during macronuclear development has been well documented in two classes of ciliates, the holotrichous ciliate *Tetrahymena* (1, 7) and the hypotrichous ciliates (reviewed in reference 16). The resulting linear macronuclear DNA fragments, which we will refer to as macronuclear chromosomes, have telomeric sequences added to their ends. Work by Preer and Preer (20) suggested that fragmentation of micronuclear chromosomes also occurs in *P. tetraurelia*.

The telomeric sequences of several ciliates have been determined. The linear macronuclear chromosomes of two holotrichs, *Tetrahymena* and *Glaucoma*, terminate with (C4A2 · T2G4) tandem repeats (4, 14, 31). Hypotrichous ciliates such as *Oxytricha* have C4A4 repeats on their macronuclear DNA ends (17). In both cases the telomeric repeats are highly homogeneous, with no sequence variation. Several lines of evidence from both holotrichs and hypotrichs support the idea that during macronuclear development telomeric repeats are added de novo to the nontelomeric sequence at the ends of newly formed linear DNA molecules (5, 15, 32). Recently, an enzyme that adds telomeric repeats de novo to a telomeric sequence primer was identified in vitro in *Tetrahymena* (11); however, this enzyme does not add telomeric repeats to a nontelomeric sequence. This raises the questions of how the developmentally controlled addition of telomeric repeats to nontelomeric DNA takes place and what *cis*-acting signals specify telomere formation. We explored this question in *P. tetraurelia* by studying different examples of macronuclear processing that create new telomeres at the A surface antigen gene locus. The A surface antigen gene is a single Mendelian gene encoding one of a family of variable surface antigens found in *P. tetraurelia* (9).

In this paper we show that developmentally controlled macronuclear telomere formation in *P. tetraurelia* has several novel features not previously seen in other ciliates. Alternative processing of the micronuclear DNA in homozygous wild-type cells creates a macronuclear telomere 8, 13, or 26 kilobases (kb) from the 3′ end of the A gene. Unlike the telomeres of other holotrichous ciliates, the telomeres of *Paramecium* consist of a random mixture of C4A2, C3A3, and rare C3A4 repeats. Variable rearrangements occur in the region in which the most proximal telomere is added, a novel finding for a ciliate telomere addition locus. The telomeric repeats are added within a specific 200- to 500-base-pair (bp) region rather than at a single precise site. This is the case not only in wild-type cells but also in an unusual *P. tetraurelia* mutant, d48 (8), in which incorrect processing of the micronuclear DNA leads to the formation of a macronuclear telomere near the 5′ end of the A gene and the loss of the DNA distal to the telomere addition site. In the d48 mutant we find no evidence for rearrangements at the A gene locus telomere. Instead, the chromosome is broken and telomeric repeats are added directly to the truncated wild-type sequence; thus, the mutant d48 creates a true terminal deletion.

MATERIALS AND METHODS

Materials. Restriction enzymes, *Bam*HI methylase, T4 DNA ligase, and Bal31 nuclease were purchased from New England BioLabs, Inc., and used in accordance with the recommendations of the manufacturer. DNA polymerase I and the Klenow fragment were purchased from Boehringer Mannheim Biochemicals and International Biotechnologies, Inc. α-^{32}P-labeled deoxynucleoside triphosphates (10 mCi/

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ml; 400 Ci/mmol or 3,000 Ci/mmol) were obtained from Amersham Corp. Nitrocellulose filters for screening libraries and Nytran filters for genomic Southern blotting were purchased from Schleicher & Schuell, Inc. (BA85; 0.45-μm-pore size).

Cultures. Kappa-free stock 51 of *P. tetraurelia* and isogenic strain d48 were obtained from the Indiana University collection and cultured as previously described (25) in wheat grass medium (Pines Distributors International, Inc.) inoculated with *Klebsiella pneumoniae*.

DNA isolation. Packed cells (0.1 to 0.4 ml) were suspended in 0.7 ml of culture medium and squirted rapidly into 2.1 ml of lysing solution (0.01 M Tris, 0.05 M sodium EDTA, 1% sodium dodecyl sulfate [pH 9.5]) at 65°C. After 20 min, 7 ml of saturated cesium chloride was added, and the DNA was purified by equilibrium centrifugation.

Library construction. Genomic DNA (5 μg) was digested with Bal31 (0.75 U) for 20 to 45 s at 30°C in 600 mM NaCl–12 mM $CaCl_2$–12 mM $MgCl_2$–20 mM Tris (pH 8.0). *Bam*HI linkers (1 μg) were ligated to the uncut genomic DNA (5 μg) at 4°C in 50 μl of 1× ligation buffer. The genomic DNA was digested with the appropriate restriction enzyme and electrophoresed on an 0.8% agarose gel. Size-selected DNA was isolated from the gel and ligated to pUC9 (for the d48 library) or λ EMBL4 (for the wild type library). For the d48 plasmid library, the DNA was methylated with *Bam*HI methylase after Bal31 digestion and before linker ligation. The wild-type library was probed with a 2.2-kb *Eco*RI fragment that includes a portion of the 3′ end of the A surface antigen gene (fragment l [see Fig. 2]) as well as a C4A2 repeat probe to differentiate between telomeric and nontelomeric clones.

Sequencing. DNA fragments were subcloned into pUC118 or pUC119 and transformed into bacterial strain MV1193. Single-stranded DNA was produced by the addition of helper phage M13K07 (J. Vieira and J. Messing, Methods Enzymol., in press). Dideoxy sequencing was performed by previously described procedures (23).

Southern blots and hybridizations. Southern blots were prepared by a modification of the Southern procedure (28) as described by Forney et al. (9). Prior to hybridization, filters were prewashed with 10× Denhardt solution–0.1% sodium dodecyl sulfate–0.2 M phosphate buffer–5× SET (1× SET is 0.15 M NaCl, 0.03 M Tris, and 2 mM EDTA) at 65°C for 1 h. The filters were prehybridized with 5× SET–0.2 M phosphate buffer–1× Denhardt solution–1% sodium dodecyl sulfate at 65 or 50°C. AFter 1 h, labeled probe was added. After 12 to 15 h, the filters were washed with 0.2× SET–0.025 M phosphate buffer–0.1% sodium PP_i–0.1% sodium dodecyl sulfate three times for 30 min each time at 65 or 50°C. The filters were air dried and exposed to Kodak X-Omat AR film at −70°C with Cronex Lightning-Plus intensifier screens.

Nick translations. Routinely, 0.25 to 0.5 μg of DNA was nick translated to a specific activity of 2×10^7 to 7×10^7 cpm/μg with 60 μCi of α-^{32}P-labeled deoxynucleoside triphosphates (18). Reactions were carried out at 15°C.

RESULTS

Location of the A surface antigen gene near a macronuclear telomere. Restriction digests of wild-type genomic DNA cut with *Pst*I or *Xho*I restriction enzymes and probed with DNA beyond the 3′ end of the A gene revealed three bands instead of the one expected band; an example is shown in Fig. 1, lane 3, in which genomic DNA was cut with *Xho*I and probed with a 2.3-kb *Eco*RI-*Bgl*II fragment approximately 6 kb from the A gene (fragment m [see Fig. 2]). Figure 1, lanes 1 and 2, show *Eco*RI and *Hin*dIII digests of total genomic DNA. Both of these digests revealed one heterogeneous band and a tight doublet that will be discussed below. When genomic DNA was first treated with Bal31 and then restricted with *Xho*I, all three *Xho*I bands were Bal31 sensitive (data not shown). Thus, the A gene is located near a macronuclear telomere. Since all cells in this study were homozygous, the three bands cannot represent different alleles. To determine whether these bands represent different-sized A gene macronuclear chromosomes or other macronuclear chromosomes that have homology to the probe, we analyzed DNA from a homozygous mutant (d12) that has a deletion of the A gene and DNA 3′ to the A gene (J. Forney and L. Epstein, unpublished data). The d12 mutation is tightly linked to the A surface antigen gene locus (21). When d12 DNA was digested with *Pst*I and probed with the 2.2-kb *Eco*RI fragment (fragment l [see Fig. 2]), none of the three telomeric bands was present (data not shown). The simultaneous absence of all three bands in the d12 mutant demonstrates that they must represent different forms of the

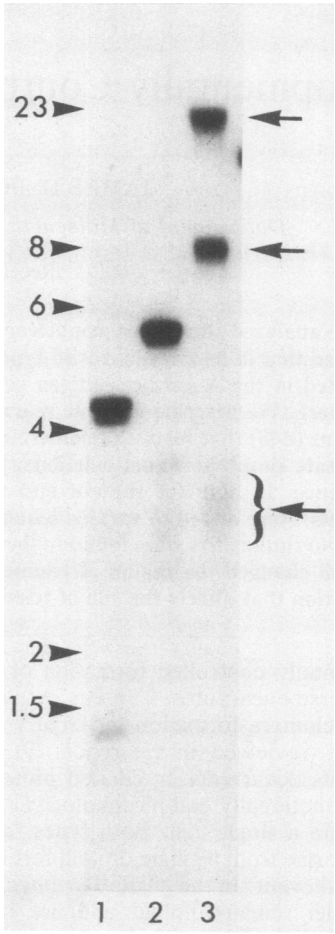

FIG. 1. Hybridization of a genomic Southern blot with the 2.3-kb *Eco*RI-*Bgl*II fragment (pEJ1). Wild-type total genomic DNA (5 μg) was digested with restriction enzymes, electrophoresed on 0.8% agarose, blotted to a Nytran membrane, and hybridized with the nick-translated 2.3-kb *Eco*RI-*Bgl*II fragment (fragment m [see Fig. 2]). The arrowheads indicate size markers in kilobases. The three arrows identify the three *Xho*I telomeric bands. Lanes: 1, *Eco*RI digest; 2, *Hin*dIII digest; 3, *Xho*I digest.

A gene macronuclear chromosome created during macronuclear development.

A map of the A surface antigen gene region and the three major telomeres is shown in Fig. 2. The A gene is located 8, 13, or 26 kb from the macronuclear telomere. The three telomeric bands appeared reproducibly as fragments of the same size in different DNA preparations. Since *P. tetraurelia* cells in cultures frequently go through self-fertilization cycles (autogamy), the macronuclear DNA preparation from each culture is derived from hundreds of independently formed macronuclei; thus, the three bands represent discrete alternate telomeres and are designated telomeres 1, 2, and 3, with telomere 1 being the closest to the A gene. It is possible that only a single type of telomere is made in each macronucleus and that the variation is a result of different telomeres being made in different developing macronuclei. We have not addressed this question.

Creation of simple terminal deletions in the wild-type A gene by the d48 mutation. In an effort to identify *cis*-acting signals specifying telomere addition in *P. tetraurelia*, we took advantage of an unusual mutation that affects the processing of the A gene macronuclear chromosome. Genomic Southern blotting data revealed that the d48 mutant has a macronuclear deletion of most of the A gene and all of its 3′-flanking DNA (Fig. 2) (8). Nevertheless, a complete wild-type copy of the A gene and its flanking sequences are present in the micronuclei of the d48 mutant. The cytoplasm of d48 controls the development of its macronucleus. When a d48 cell is mated to a wild-type cell, the macronuclei that develop in the wild-type cytoplasm all contain the complete wild-type A gene, and the macronuclei that develop in the d48 cytoplasm have deletions of the A gene. This is true despite the fact that both cells have genetically identical micronuclei. The ability of the complete A gene to be incorporated into the new macronucleus is determined by cytoplasmic factors produced by the old macronucleus of the parental cell (12). These results suggest that a *trans*-acting factor specifically affects processing of the A gene locus during macronuclear development.

As a first step in understanding the molecular nature of this mutation, we cloned the mutant A gene macronuclear telomere from d48. A library was made by using a procedure designed to enrich for telomeric clones (see Materials and Methods). This library was screened with the 3.7-kb *Hin*dIII fragment that includes the 5′ end of the A surface antigen gene as well as 3 kb upstream of the gene (fragment k [see Fig. 2]). Six positive clones were isolated, and four were chosen for further study. The DNA near the site of telomere addition was sequenced. The sequences of the four clones, aligned with the previously published wild-type sequence (21), are shown in Fig. 3. Several important points emerge from this analysis. First, the subtelomeric DNA from each clone is identical to the wild-type DNA until the point of telomere repeat addition; thus, d48 creates a simple terminal deletion of the A gene macronuclear chromosome. Second, the point of telomere repeat addition is variable. The distance between the most proximal and most distal telomere addition sites is 284 bp. Third, the telomeric sequence consists of a combination of G4T2 and G3T3 repeats. While it is clear that these two types of repeats constitute the majority of the telomeric sequence, one clone (d48-4) also contained three tandem G3T4 repeats. It seems unlikely that these repeats are the result of a cloning artifact; they probably represent a minor class of telomeric repeats in this organism. Finally, three of the telomere addition sites, represented by clones d48-1, d48-6, and d48-8, are within the relatively G+C-rich coding region of the A surface antigen gene. The *Paramecium* genome is 29% G+C (24), while the coding region in this area is 36% G+C.

Occurrence of a DNA rearrangement at wild-type telomere 1. We compared the d48 mutant telomere addition site to wild-type telomere 1, which is 8 kb from the A gene. Telomere 1 was analyzed by cloning several examples of this telomere (λ SA4 clones) as well as the 13-kb *Bgl*II restriction fragment overlapping telomere 1 (λ SA3 clones) (Fig. 2). The sequence of the SA4 telomeric clones was determined from the *Sac*I site (Fig. 2) into the telomeric repeats. Analysis of the wild-type telomere sequences (labeled SA4-1, SA4-3, SA4-4, and SA4-6 in Fig. 4) demonstrated an even greater heterogeneity of telomere addition sites than that seen in the d48 mutant. The distance between the most proximal and distal telomere addition sites was 427 ± 2 bp. Although by comparison with longer telomeres the point at which G4T2 and G3T3 repeats were added was clear for clones SA4-4, SA4-1, and SA4-6, we were unable to resolve the exact point of telomere addition for SA4-3. This was because it was not

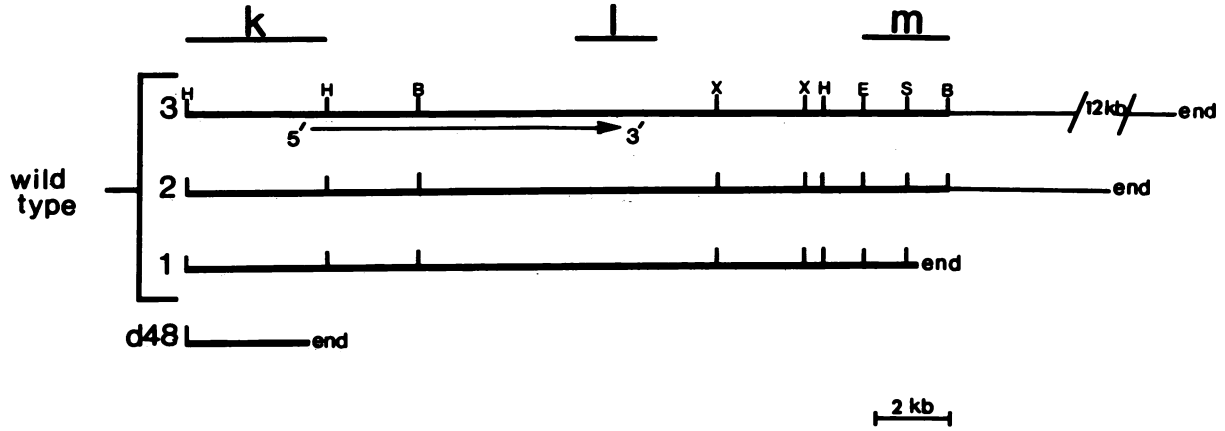

FIG. 2. Map of the A surface antigen gene region. The three alternate wild-type macronuclear chromosomes and the d48 mutant telomere addition site are shown. 1, 2, and 3 indicate macronuclear chromosomes with telomeres 1, 2, and 3, respectively. The A surface antigen gene transcript is indicated by an arrow. k, l, and m indicate fragments used as probes for various experiments; k, 3.7-kb *Hin*dIII fragment; l, 2.2-kb *Eco*RI fragment; m, 2.3-kb *Eco*RI-*Bgl*II fragment (pEJ1). The DNA distal to the right-hand *Bgl*II site has not been cloned. Only selected *Hin*dIII, *Eco*RI, and *Sac*I sites are shown. B, *Bgl*II; E, *Eco*RI; S, *Sac*I; X, *Xho*I; H, *Hin*dIII.

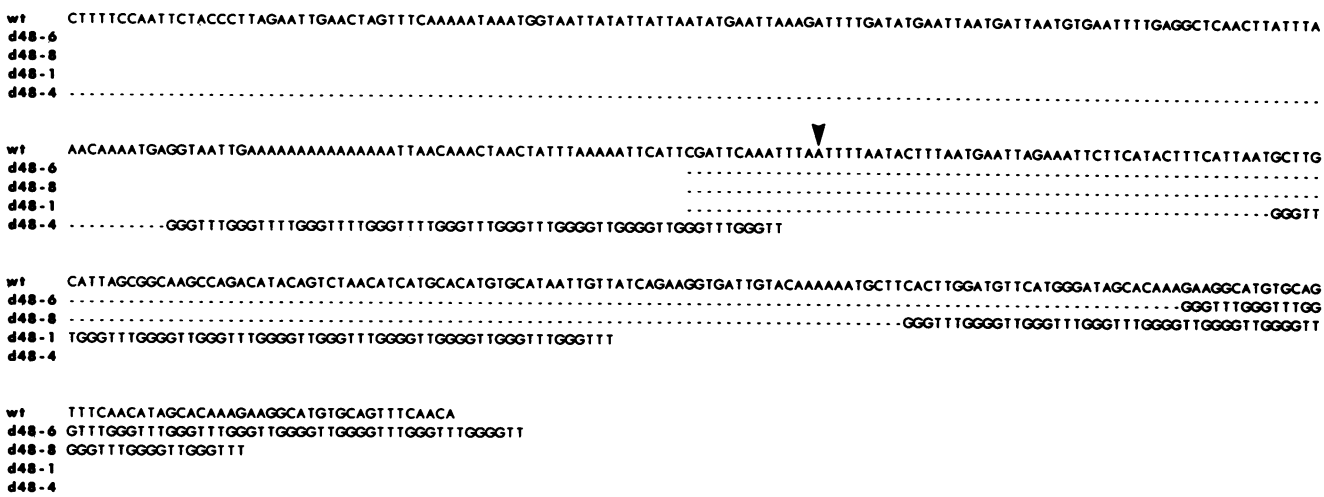

FIG. 3. Comparison of sequences of d48 mutant telomere clones with the wild-type A gene sequence. The wild-type sequence is shown on the top line (wt). The four mutant telomere clones (d48-1, d48-4, d48-6, and d48-8), shown in the 5′-to-3′ orientation, are aligned by their unique sequences. The dashes represent bases identical to the wild-type bases, and the blank spaces in the subtelomeric regions indicate DNA that was not sequenced. The sequences of the first 10 telomeric repeat units are shown for each telomere clone. The arrowhead identifies the start of transcription of the A surface antigen gene (21).

possible from these data to determine how many G and T residues represented a unique chromosomal sequence or a de novo-added telomere sequence.

We anticipated that by sequencing the DNA spanning the position of telomere 1, we could identify *cis*-acting sequences important for telomere addition and compare them with sequences surrounding the d48 mutant telomere sites. The most distal *Eco*RI-*Bgl*II fragment from SA3-1 (fragment m [see Fig. 2]) was subcloned into pUC9 and named pEJ1 (for end junction 1). The pEJ1 insert will be referred to as

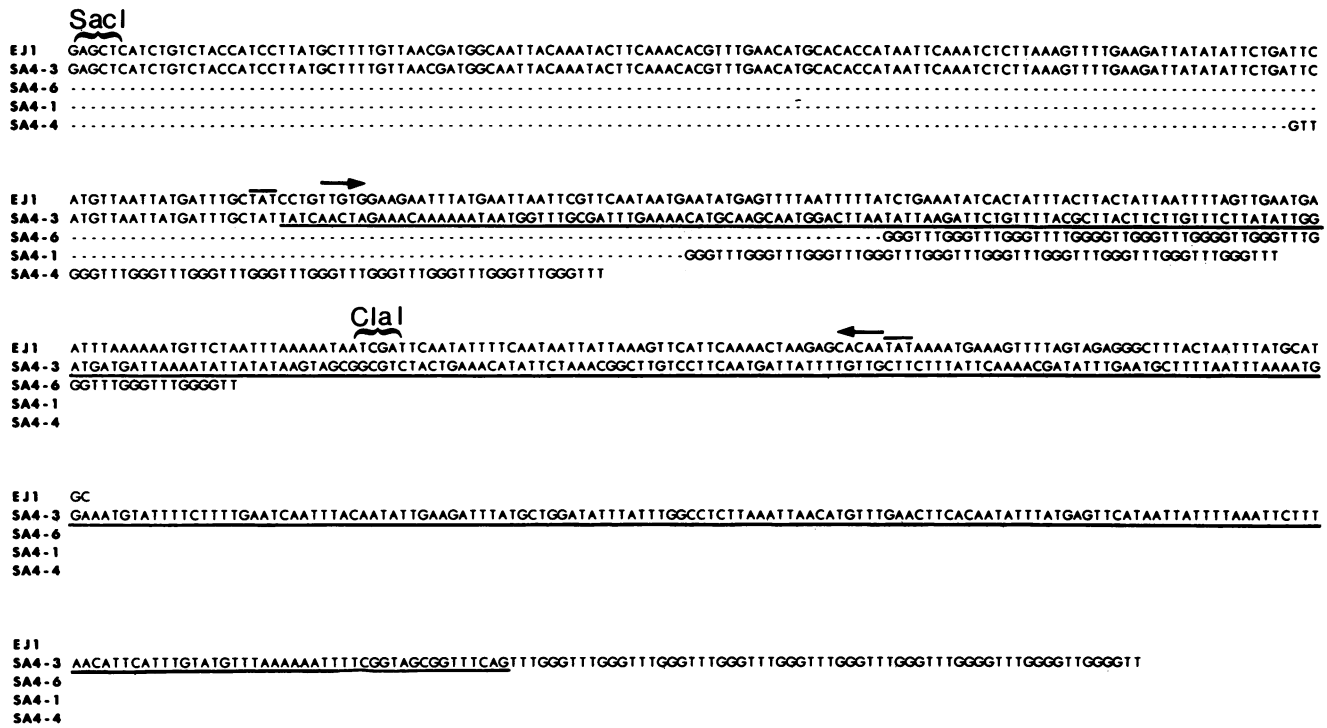

FIG. 4. Comparison of sequences of wild-type telomeres at A gene locus telomere 1 with the sequence of a chromosome that uses telomere 2 or 3. The top line (labeled EJ1) is the sequence of a macronuclear chromosome that uses telomere 2 or 3. The 3-bp direct repeats that flank the deletion found in clone pEJ1a are overlined. The arrows indicate the inverted repeats found adjacent to the direct repeats. The sequences of four different wild-type telomere 1 clones, shown in the 5′-to-3′ orientation, are labeled SA4-1, SA4-3, SA4-4 and SA4-6. The dashes indicate nucleotides identical to SA4-3 nucleotides. The portion of SA4-3 that is not homologous to EJ1 is underlined from the point of divergence to the approximate site of telomere repeat addition. The *Sac*I and *Cla*I sites labeled on EJ1 are discussed in the text. The sequences of the first 10 telomeric repeat units are shown for each telomere clone.

junction DNA. The first 375 bp distal to the *Sac*I site of pEJ1 were sequenced (labeled EJ1 in Fig. 4). However, in contrast to the A gene telomeres in the d48 mutant, the subtelomeric DNA of the wild-type telomeres was not completely colinear with the sequence of pEJ1. Three of the telomeric clones (SA4-1, SA4-3, and SA4-6 [Fig. 4]) had the same sequence as the junction DNA up to a position 180 bp distal to the *Sac*I site. At this nucleotide, the sequence of the three telomeric clones completely diverged from the junction DNA (underlined sequence in Fig. 4). We shall refer to this sequence, not found in pEJ1, as "extra" DNA. In the fourth telomeric clone (SA4-4), telomeric repeats were added proximal to the site of divergence.

To determine whether the extra DNA was located at A gene telomeres 2 and 3 as well as telomere 1, we hybridized it to genomic Southern blots (data not shown). The Southern blot data revealed that the extra DNA sequence was found in several macronuclear genomic locations but was not highly repetitive. The presence of several bands in the genomic digests made it difficult to determine whether telomere 2 or 3 contained extra DNA. The extra DNA did not hybridize to SA3-1, indicating that it was not located within 1 kb of telomere 1 on the longer A gene macronuclear chromosomes (data not shown).

Variable DNA rearrangements associated with telomere 1. Because specific rearrangements had not previously been seen at telomere addition sites in other ciliates, we were interested in any other variable rearrangements that took place in the telomere 1 region. As previously mentioned, all DNA in these experiments came from homozygous cell lines. Thus, any restriction site polymorphisms in macronuclear DNA must represent differential processing during macronuclear development. Genomic Southern blots probed with pEJ1 revealed hybridization with a doublet in *Eco*RI-*Bgl*II, *Eco*RI, and *Hin*dIII digests (*Eco*RI and *Hin*dIII digests are shown in Fig. 1). Both bands of the doublet were internal fragments and represented A gene macronuclear restriction fragments, based on their absence in genomic DNA from d48 and restriction fragments, based on their absence in genomic DNA from d48 and d12 (an A gene deletion mutant) (data not shown). These data were consistent with a small deletion in a subpopulation of the DNA molecules in the region of telomere 1. Analysis of five SA3 clones revealed two classes of clones that had identical restriction maps except for a small size difference (approximately 200 bp) in the most distal *Eco*RI-*Bgl*II fragment (fragment m [see Fig. 2]). All clones appeared to fall into one of the two classes, with no intermediate restriction fragment sizes. The previously sequenced junction DNA (pEJ1) came from the larger size class. One clone (pEJ1a) was chosen from the smaller size class for further analysis. The restriction map of pEJ1a was identical to that of pEJ1 except for the 200-bp size difference and the loss of a *Cla*I restriction site located within 100 bp of the site of the telomeric DNA rearrangement (Fig. 4). The sequence of pEJ1a distal to the *Sac*I site was determined. The pEJ1a clone had a small deletion of 190 bp, as compared with pEJ1. One endpoint of this deletion was precisely at the nucleotide at which the extra DNA was located in the telomere 1 clones SA4-4, SA4-1, and SA4-6 (Fig. 4). The 190-bp deletion was bounded by a 3-bp direct repeat in pEJ1, one copy of which was retained in the deleted version (pEJ1a) of the macronuclear chromosome. The internal region of the deleted DNA also contained a pair of 5-bp inverted repeats, one four nucleotides inside one direct repeat and one immediately adjacent to the other direct repeat (Fig. 4).

DISCUSSION

Chromosomal location of variable surface antigens. We have shown that the variable A surface antigen gene in *P. tetraurelia* lies near a macronuclear telomere. The G surface antigen gene in *P. primaurelia* is also located near a macronuclear telomere (3, 19), but it is not yet clear if the telomeric location of variable surface antigen genes is a general feature in *Paramecium* species, as the H surface antigen gene in *P. tetraurelia* is at least 18 kb from a macronuclear telomere (10). It is of interest that the expressed variable surface antigen genes in trypanosomes are similarly located within several kilobases of telomeres (6). Transcription of trypanosome variable surface antigen genes is toward the telomere; the same is true for the A gene. Mutually exclusive expression of surface antigen genes occurs in both systems, but unlike the situation in trypanosomes, no rearrangements are associated with changes in *Paramecium* surface antigen expression (9). The permanent telomeric location of *Paramecium* surface antigen genes could be analogous to the multiple variable surface antigen expression sites found in trypanosomes.

Creation of a true terminal deletion in the macronucleus by the d48 mutation. The analysis of the d48 mutant telomere has shown that altered macronuclear processing of the A gene region leads to a broken DNA end that is "healed" solely by the addition of telomeric repeats and not by recombination of a short segment of a wild-type telomere onto the A gene macronuclear chromosome. The latter possibility was not ruled out in previous studies of d48 (8). The current model of the effect of d48 on the processing of the A surface antigen gene proposes that the d48 mutant lacks some product, made by the macronucleus, that is responsible for the correct processing of the A surface antigen gene chromosome. The results reported in this paper show that without this product the chromosome is broken at the 5′ end of the A gene and that telomeric repeats are added directly to the broken ends. The nature of the product and its precise role in processing are unknown. A trichocyst discharge mutation and mating type inheritance in *P. tetraurelia* are also controlled by factors, made by the old macronucleus, that control the formation of the new macronucleus in an analogous fashion (26, 27). The similarity of the genetics in all three examples of non-Mendelian inheritance makes it likely that they have a common molecular basis.

Sequence requirements for telomere addition sites. In both wild-type and d48 mutant cells, the telomere addition site is not a single specific sequence. Instead, the sequences that can serve as alternate substrates for telomere addition are located within a specific region of a few hundred base pairs. By both computer and visual searches we were unable to find any primary sequence, on either the 5′ or 3′ side of the site, that correlated with telomere addition, suggesting that few constraints exist on the sequence that can be used for telomere addition during macronuclear development. Although there is no evidence for a specific telomere addition sequence, this study shows that specific telomere addition regions exist. The DNA preparations from the wild-type cultures represent large numbers of independent macronuclear formation events, yet the same three regions are always used for telomere addition. Likewise, the d48 mutation creates a single telomere addition region at the 5′ end of the A surface antigen gene. Although the full extent of the heterogeneity in the telomere addition regions is not known, the telomeric restriction fragments in genomic Southern blots showed limited breadth (Fig. 1) (8). Since some of the

heterogeneity may represent variable numbers of telomeric repeats, it is likely that the telomere addition regions are not much larger than those represented by the cloned examples.

Randomly selected macronuclear telomeres from *Tetrahymena thermophila* have telomeric repeats added to very A+T-rich (83 to 90%) regions (34; E. Spangler and E. Blackburn, unpublished data). The sequences of four clones containing the telomere near the G surface antigen gene of *P. primaurelia* have been reported; again, the telomeric repeats are found adjacent to A+T-rich sequences (3). In contrast, unusually A+T-rich subtelomeric sequences were not found in the *P. tetraurelia* telomere addition sites reported in this paper. Three of the d48 mutant telomeres and the wild-type telomeres are located adjacent to DNA sequences with G+C compositions of 36 and 27%, respectively. The G+C content of *P. tetraurelia* total genomic DNA is only 29%, making it difficult to argue that these sequences are unusually A+T rich. The four cloned telomeres from *P. primaurelia* also show localized telomere site heterogeneity, as we have shown here for *P. tetraurelia*. Yet, unlike the three telomeres at the A surface antigen gene locus of *P. tetraurelia*, the G surface antigen gene locus has only one telomere addition region.

The inability to find a precise sequence that is essential for telomere addition may mean that during a specific period in ciliate macronuclear development, any linear DNA will have telomeric repeats added to its free ends. Although the telomere repeat addition enzyme identified by Greider and Blackburn only adds telomeric repeats to preexisting telomeric repeats (11), the enzyme might be activated by an additional factor to add telomeric repeats to a nontelomeric sequence. The overlapping sequence of wild-type and d48 mutant telomere clones allows us to unambiguously identify in seven examples the first telomeric repeat that is added de novo to the chromosomal sequence. Interestingly, in five of them three G residues are the first nucleotides added, and in all of them the first base added is a G residue. The sequence data also show that the first repeat added de novo is not primed by even a single G or T residue. Together, these data establish that no sequence constraints exist for telomere addition.

The regional specificity of telomere addition seen in *Paramecium* could be a result of particular DNA conformations that are recognized and broken by an endonuclease lacking strong sequence specificity. Alternatively, a specific sequence may be present in the *Paramecium* micronucleus more than 500 bp from the site of macronuclear telomere addition, and the heterogeneity could be a result of variable extents of exonucleolytic trimming from the broken end. Similarly, no conserved micronuclear sequence has been found near macronuclear telomere addition sites in the hypotrichous ciliate *Oxytricha* (16). In *T. thermophila*, a conserved 15-bp sequence found at micronuclear chromosome breakage sites has recently been identified (32). The conserved sequence is present in the eliminated portion of the micronuclear chromosome. In contrast to the findings in *Paramecium* and *Oxytricha*, the two *Tetrahymena* examples previously studied show that the conserved sequence is within 25 to 30 bp of the site of macronuclear telomere addition. Thus, it is possible that *T. thermophila* is unusual in its primary sequence requirements for micronuclear chromosome breakage.

Alternate developmental fates of a sequence found near a wild-type telomere. The 190-bp deletion reported here is the first example of a developmentally controlled deletion in *Paramecium*. The endpoints of the deleted segment near telomere 1 have DNA sequence characteristics similar to those of internal deletions found in *Oxytricha nova*, which have short direct repeats (3 to 4 bp) adjacent to inverted repeats (15, 22). Direct repeats have also been found at the boundaries of an eliminated sequence in *T. thermophila* (2). Interestingly, the 190-bp deletion occurs only when telomere 1 is not formed and only in some of the macronuclear chromosomes with telomere 2 or 3. Furthermore, the left

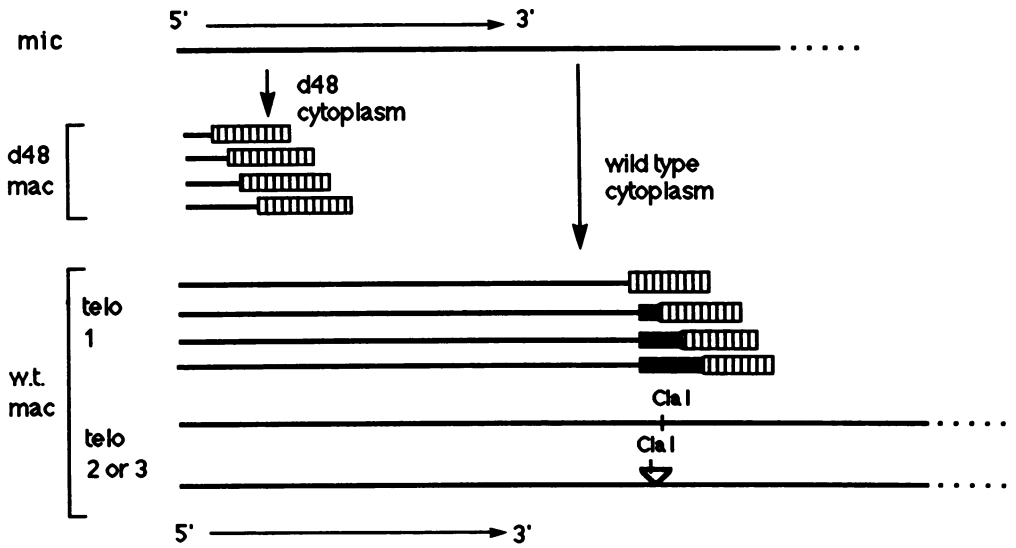

FIG. 5. Summary diagram of macronuclear telomere formation at the A gene locus in wild-type and mutant cells. This diagram incorporates our knowledge of macronuclear telomere addition sites as well as the genetics of wild-type and d48 strains. The top line represents the A gene locus in the micronucleus. The arrows represent the process of macronuclear development. In d48 cytoplasm, the micronuclear DNA is broken and telomeric repeats are added to a region at the 5′ end of the A surface antigen gene (the A gene transcript is represented by 5′→3′). In wild-type cytoplasm, the micronuclear DNA may undergo a 190-bp deletion (represented by ▽) when telomeric repeats are added at telomeres 2 or 3. The *Cla*I restriction site in the 190-bp deletion is indicated. ▮, Telomeric repeats; ■, extra DNA; mic, micronucleus; mac, macronucleus; w.t., wild type.

end of the deletion is precisely the same as the junction of the extra DNA at telomere 1. Variable rearrangements such as this appear to be unusual in *P. tetraurelia*. Despite extensive searches carried out previously (9), no variable rearrangements were found near the A and other surface antigen genes.

Our results show that the formation of the wild-type telomere near the A gene is more complex than the simple terminal deletion found in d48. Figure 5 summarizes diagrammatically the relationships between the various telomeres formed at the A gene locus in both wild-type and d48 cells. This is the first example in ciliates of a variable rearrangement that occurs at a telomeric location. The other examples of variable rearrangement involve sequences solely at internal chromosomal locations (13, 15, 30). The data presented here are most simply explained if the extra DNA at telomere 1 is the result of a recombination event followed by the addition of telomeric repeats. The existence of the shorter telomere 1 clones which lack extra DNA suggests that telomeres are added after the recombination event. Otherwise, two different mechanisms would be necessary: one that adds telomeres to unrearranged telomere 1 and one that adds telomeres to extra DNA. An alternative but less likely possibility is that the extra DNA actually represents the unrearranged micronuclear DNA and that the DNA present in the junction fragment (pEJ1) is the rearranged version of the macronuclear chromosome. In this model extra DNA is located at the primary telomere addition site, but if a rearrangement event takes place it is destroyed and an alternate site farther away from the A surface antigen gene is used to produce telomere 2 or 3. We do not favor this model because sequence analysis of the 190-bp deletion showed direct and inverted repeats at its ends in an arrangement typical of deleted segments of DNA in ciliates (2, 15, 22). In contrast, sequence analysis of the left border of the extra DNA revealed no counterpart of the inverted repeat sequence at the right border of the 190-bp deletion (Fig. 4). Such a sequence would be expected if the extra DNA represents the unrearranged micronuclear sequence. Regardless of the pathway of A gene telomere formation, it appears to be unusual in ciliates. None of the examples of macronuclear telomere formation in *Tetrahymena* or *Oxytricha* involve rearrangements at the telomere addition sites (16, 32, 33).

ACKNOWLEDGMENTS

We thank John Preer, in whose laboratory J.D.F. made the initial observation that the A surface antigen gene was located near a macronuclear telomere.

This research was supported by Public Health Service grant GM26259 from the National Institutes of Health to E.H.B. and by a Helen Hay Whitney postdoctoral fellowship to J.D.F.

ADDENDUM IN PROOF

Recent work by Herrick et al. (G. Herrick, D. Hunter, K. Williams, and K. Kotter, Genes Dev. 1:1047–1058, 1987) showed that alternate processing of a micronuclear sequence occurs during macronuclear development in *Oxytricha fallax*. Microheterogeneity in the site of telomere repeat addition was also observed in this study.

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