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New Telomere Formation Coupled with Site-Specific Chromosome Breakage in *Tetrahymena thermophila*

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Programmed chromosome breakage occurs in many ciliated protozoa and is accompanied by efficient new telomere formation. In this study, we have investigated the relationship between programmed chromosome breakage and telomere formation in *Tetrahymena thermophila*. Using specially constructed DNA clones containing the breakage signal Cbs in transformation studies, we have determined the locations of telomere addition around the breakage sites. They occur at variable positions, over 90% of which are within a small region (less than 30 bp) starting 4 bp from Cbs. This distribution is independent of the nucleotide sequence in the region or of the orientation of Cbs. In five of six cases determined, these sites occur at or before a T, and in the remaining case, the site occurs at or before a G. When sequences devoid of G or T are placed in this region, telomere addition still occurs within the region to maintain a similar distance relationship with Cbs. This efficient end healing process appears to be associated specifically with Cbs-directed breakage, since it does not occur when DNA ends are generated by restriction enzyme digestion. These results suggest a strong mechanistic link between chromosome breakage and telomere formation.

Most eukaryotic chromosomes contain special structures at their ends (telomeres) which are critical for chromosome stability and propagation (41). Broken chromosomes lacking telomeres are highly unstable and are usually lost or recombined with other chromosomes. In rare cases, telomeres can be generated de novo to stabilize broken ends (21, 27). The details of this end healing process are not entirely clear. In some organisms, including ascarid nematodes (30) and ciliated protozoa (25, 33), chromosome breakage occurs in many genomic locations in a precisely regulated manner during somatic nuclear differentiation. In this remarkable process, the broken ends are efficiently healed, and the chromosome fragments thus generated are stably propagated throughout the organism's somatic life. The high efficiency of this process raises an interesting question regarding its regulation and provides a unique opportunity to study its underlying mechanism.

The ciliate *Tetrahymena thermophila* is a favorable organism for the study of chromosome breakage. Like most ciliates, it contains a micronucleus (germ line nucleus) and a macronucleus (somatic nucleus). During conjugation, the micronucleus undergoes meiosis, cross-fertilization, and mitosis to generate the precursors for the new micro- and macronuclei of the following vegetative life. The old macronucleus degenerates during this process (7). Development of the new macronucleus involves a series of well-regulated DNA rearrangement processes which result in the formation of a drastically altered somatic genome (33). One of these processes is chromosome breakage, which occurs at approximately 200 specific sites and produces chromosome fragments averaging 800 kb in size. These chromosome fragments are propagated indefinitely in the mature macronucleus, which divides by an unusual amitotic process during vegetative growth.

Chromosome breakage in *T. thermophila* is accompanied by the formation of new telomeres. This conclusion is based initially on the observation that many, and probably all, of the telomeric sequences present at macronuclear DNA termini are not derived from the germ line genome (34). Analysis of sequences at several specific breakage sites has confirmed this view (8, 20, 37, 39). These sites contain a common 15-nucleotide sequence, Cbs, which serves as the signal for chromosome breakage. A DNA segment including this sequence and 15 to 30 nucleotides from both sides is lost after breakage, and new telomeric sequences are formed at the ends of the remaining DNAs (37). The relationships among DNA cleavage, DNA loss, and telomeric sequence addition are not clear.

This laboratory had previously developed a transformation system for the analysis of chromosome breakage in *T. thermophila* which uses the rRNA gene as a selectable marker (31, 35). In the micronucleus of *T. thermophila* the gene coding for rRNA (rDNA) is present as a single-copy gene and is flanked by copies of Cbs (38). This gene is excised from the chromosome by breakage and becomes highly amplified in the macronucleus (32). Cloned copies of the micronuclear rDNA are accurately processed when introduced into developing macronuclei (35). With such a system, we had shown previously that Cbs is necessary and sufficient for chromosome breakage in *T. thermophila*. Deletion of Cbs abolishes breakage, and insertion of Cbs at new sites induces breakage to occur at these sites (36). In this study, we have further analyzed the link between telomere addition and chromosome breakage. We have found a distinct distance relationship between the sites of telomere addition and the location of Cbs. This relationship is not affected by the sequence context of the region or the orientation of Cbs. In addition, we have shown that efficient end healing is probably specific to ends generated by breakage at Cbs, since it does not occur at ends produced by restriction enzyme cutting. These results offer important insights into the mechanism linking programmed chromosome breakage and telomere addition.

MATERIALS AND METHODS

***Tetrahymena* stocks, culturing conditions, and transformation methods.** *T. thermophila* inbreeding line B strains CU427 and CU428 were obtained from Peter Bruns and were used throughout this study. Cells were grown in enriched peptone medium with constant aeration as described previously (14). For trans-

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formation studies, these two strains were starved and mated, and DNAs were injected into developing macronuclei by a method described previously (35). For each DNA sample, approximately 200 to 800 cells were injected. The injected cells were cloned and grown for approximately 13 generations before they were replicated into media containing paromomycin (Humitin; Park-Davis) to identify transformants. The resistant clones were grown for another 10 to 15 generations in paromomycin-containing medium before being harvested for DNA analysis.

DNA analysis. DNA was isolated from *T. thermophila* by a phenol extraction method described previously (1). Restriction enzyme digestion, agarose gel electrophoresis, gel blotting, and hybridization were carried out by standard published methods (26). Oligonucleotides were synthesized with an Applied Biosystems automated DNA synthesizer in the Fred Hutchinson Cancer Research Center. Oligonucleotides were radiolabeled at the 5′ end with ^{32}P by polynucleotide kinase before being used as hybridization probes. The oligonucleotides used for hybridization are rdn9757, which has the sequence 5′-CTACGCTTA GATTTTAACTTTATCCC, and rdn10026, which has the sequence 5′-ACAAAAAACCCTTCTAAAAG. The relative amounts of radioactivity in individual hybridization bands were determined with a PhosphorImager 400A (Molecular Dynamics, Sunnyvale, Calif.). These values were used for calculating the distributions of telomere addition sites in each injected DNA. To determine the sequence at rDNA termini, whole-cell DNA from the relevant transformed line was used as a template for PCR. One of the primers used matched the telomeric DNA sequence (5′-GGGGTTGGGGTTGGGGTT), and the other matched the rDNA sequence near its 3′ end (rdn9757 or rdn10026). The amplified DNA was cloned and sequenced by the chain termination method with either Sequenase (U.S. Biochemical Corp.) or *Taq* polymerase (Promega).

Construction of DNA clones. Both clones FA103 and FANA3 are derivatives of clone Tt94701, which contains the micronuclear rDNA from a C3 inbreeding line with a dominant mutation in the coding region for paromomycin resistance (35). Tt94701 was modified to generate D3/5-24 by removal of both rDNA flanking regions. It also contains a copy of Cbs inserted at the truncated 3′ terminus of the rDNA (36). This clone was modified by being cut with *Kpn*I and *Sma*I, treated with Klenow enzyme, and self-ligated in the presence of a *Sma*I linker (5′-CCCCGGGG) such that the inserted Cbs was replaced with a *Sma*I site. In addition, the 5′ end and the adjacent flanking sequence of the rDNA were restored in this clone by replacement of its *Mlu*I-*Xho*I fragment with the same fragment from clone 947-400 (36) to generate clone FA103. Clone FANA3 was constructed by insertion of a polylinker into the *Dde*I site at position 10144 of clone 947-400 by a two-step method described previously (29). An extra 15-bp sequence of unknown origin was also inserted into this site during cloning.

To generate clone FA069, clone FA103 was cut at the unique *Sal*I and *Sma*I sites, and a polylinker containing Cbs was inserted. To generate FA142 and FA152, clone FANA3 was cut at the *Sma*I site and two different polylinkers, both containing Cbs, were inserted. To generate clones with AC-rich or GT-rich sequence inserts, clone FA142 was cut at the unique *Sma*I site and blunt-ended double-stranded oligonucleotides with the sequences 5′-GGGACAAA AAAAAAAAAAAGG and 5′-$(GGGGTTTTT)_4$ were inserted in either orientation. The sequences and orientations of the inserts were determined by nucleotide sequencing. These sequences differed slightly from those expected, possibly because of sequence alteration during cloning.

RESULTS

Telomere addition at chromosome breakage sites. Chromosome breakage occurs in *T. thermophila* at sites containing the recognition sequence Cbs and produces free ends that contain new telomeric sequences (36). To facilitate the analysis of these new ends, we generated the following plasmid constructs for transformation studies (Fig. 1). Clone FANA3 contained a synthetic linker with multiple restriction sites in the 3′ spacer region of the micronuclear rDNA (position 10144; all rDNA nucleotide positions used in this study follow the sequence published in reference 10). Clones FA142 and FA152 were modified from clone FANA3 by insertion of a copy of Cbs embedded in two different linkers in opposite orientations into the linker region. Clone FA103 contained a different linker at a different location (position 9810) within the 3′ spacer region of the micronuclear rDNA. In addition, it lacked all of the 3′ spacer and the flanking sequence downstream from this position. Clone FA069 was modified from clone FA103 by insertion of a copy of Cbs into the linker region. Clones FA069, FA142, and FA152 are expected to generate rDNA molecules with truncated termini after they are introduced into developing cells, if breakage indeed occurs at the inserted Cbs. The presence of multiple restriction sites next to Cbs facilitates the analysis of telomere addition sites in mixed populations of transforming molecules by restriction mapping.

These cloned DNAs were injected into developing macronuclei of conjugating *T. thermophila* organisms, and paromomycin-resistant transformants were obtained at typical rates (1 to 3%). DNAs from four transformants injected with clone FA069 DNA were pooled and analyzed by Southern hybridization. Pooled DNA samples were used because they better represent the distributions of telomere addition sites. An oligonucleotide (rdn9757) which contained an rDNA sequence from a region upstream of the polylinker (from nucleotide positions 9757 to 9782) was used as a probe. Figure 2A illustrates this approach. To verify the completeness of restriction digestion, a plasmid DNA containing these restriction sites was mixed with the samples to serve as an internal control. After digestion with *Dra*I, which cut outside of the polylinker region, the transforming rDNA produced a heterogeneous population of fragments characteristic of fragments containing telomeric DNA (Fig. 2B). Their average size of 0.51 kb was in good agreement with the expected size if breakage occurred at or near the inserted Cbs and if telomeres were added to the broken ends (Fig. 2). If breakage failed to occur, a 0.39-kb *Dra*I fragment should be generated. This fragment was not detected (Fig. 2B, lane 2). This result confirms the earlier observation that Cbs is a sufficient sequence signal for chromosome breakage to occur in *T. thermophila*. A group of restriction enzymes that cut within the polylinker were then used, in addition to *Dra*I, to fine-map the positions at which telomere addition occurred. *Hpa*I, *Eco*RI, and *Cla*I digestion converted the broad band to a sharp band with a size of around 0.19 kb, indicating the presence of these sites in most of the telomeric fragments. *Xba*I was able to digest the majority (about 78%) of the molecules, whereas *Xho*I digested only a small fraction (about 14%) of them. Thus, the majority of the termini contained the *Xba*I site but not the *Xho*I site. In contrast, *Sma*I, *Sal*I, and *Hin*dIII failed to digest these molecules, indicating the absence of these sites from the fragments. By measuring the relative abundance of the hybridizing materials in the 0.51-kb region and the 0.19-kb band in each lane, we estimated that the majority of the molecules (64%, the difference between 78 and 14%) terminated within the *Xho*I site, and the rest terminated roughly equally within the two flanking sites (Fig. 2B). *Dra*I digestion of the host rDNA was expected to produce a 0.31-kb fragment detectable with this probe (Fig. 2B, lane 1). This fragment was present in various amounts in the transformed lines, and as expected, was not cut by any of the secondary enzymes used (Fig. 2B, lanes 2 to 10). The presence of some host rDNA was common in this type of transformation study (29, 35, 36).

The analysis described above was performed with DNAs pooled from four transformants. Since each transformant was injected with hundreds to thousands of DNA molecules, it is likely to contain a mixture of independently processed rDNAs. In agreement with this assessment, analysis of individual transformants has revealed degrees of sequence heterogeneity similar to that observed when pooled DNA was used (data not shown).

To determine if the local sequence context affected the pattern of telomere addition, we analyzed DNAs pooled from eight transformants that had been injected with FA142 DNA and six transformants that had been injected with FA152 DNA. As shown in Fig. 1, the location of the inserted Cbs in these clones was different from that in clone FA069. In addition, the orientations of Cbs and its immediate flanking sequence are different between these two clones. Again, the telomere addition sites in these transformants were clustered within a small

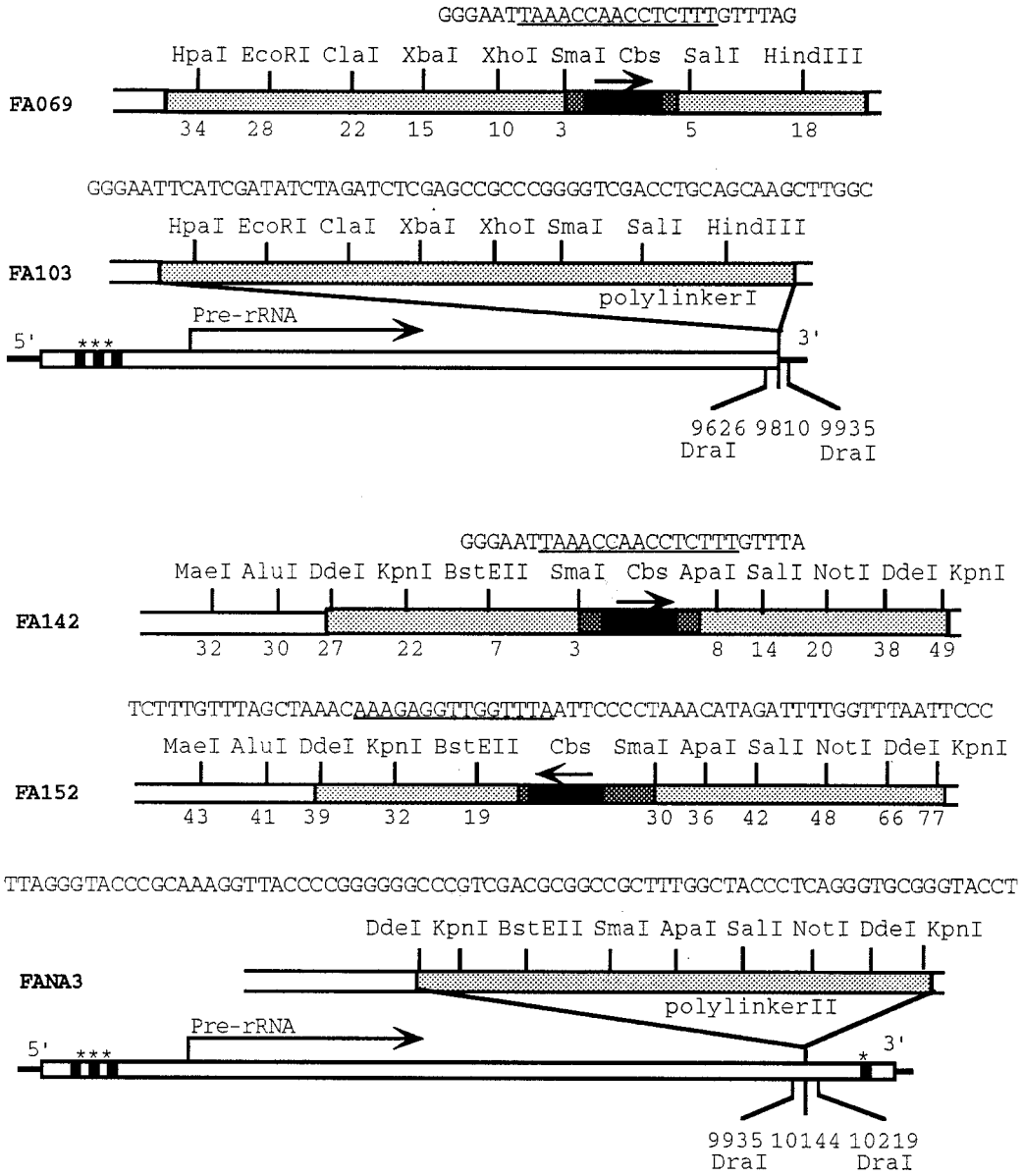

FIG. 1. Structure of the rDNA vectors and their Cbs insertions. The micronuclear rDNA clones used in this study are diagrammed to show only their relevant features. The open bars represent *T. thermophila* micronuclear rDNA and immediate flanking sequences. The arrows above indicate the orientation of transcription. The solid squares with asterisks above them are native copies of Cbs which flank this gene. The thick lines next to the *Tetrahymena* sequences represent *E. coli* plasmid sequences. The numbers below the bars indicate the nucleotide positions of the corresponding macronuclear rDNA sequences. The two *Dra*I sites immediately flanking the insertions are indicated. The shaded bars represent synthetic oligonucleotides, which are inserted at the locations indicated. The names of the clones with the particular inserts are indicated to the left. For clones FA069, FA142, and FA152, only the regions with the polylinker are shown. The restriction sites present in the inserts are indicated above, and the distances (in numbers of nucleotides) from these sites to the Cbs in these inserts are indicated below the bars. The Cbs insertions are marked by the arrowheads above the solid boxes to indicate the orientation (from the 5′ end) of the C-rich strand of Cbs. The *Alu*I and *Mae*I sites shown in clones FA142 and FA152 are outside of the linker in the neighboring rDNA sequence. The sequences of these insertions are shown above their respective restriction maps. The sequence of Cbs is underlined.

region a short distance from Cbs, although their exact distribution varied slightly (Fig. 2C and D). From these studies, we conclude that telomere addition occurs at various locations within a small region a short distance away from Cbs. Neither the orientation of Cbs nor its immediate neighboring sequence has significant influence over this basic pattern of distribution.

Nucleotide preferences at telomere addition sites. To determine the exact location of telomere addition, we amplified, cloned, and sequenced the DNAs at these free ends. Two independent *Tetrahymena* lines transformed with each DNA clone were analyzed. In agreement with the restriction mapping studies, the addition sites were scattered within a small region a short distance away from Cbs (Fig. 3). Although these sites did not share an apparent consensus sequence, they were not entirely random. In five of six cases analyzed, addition occurred at or before a T, and in the remaining case, addition occurred at or before a G. The ambiguity was due to the presence of the same nucleotide in both the injected DNA and the telomeric sequence. For instance, the T in position −16 of the first clone derived from clone FA069 could be the last

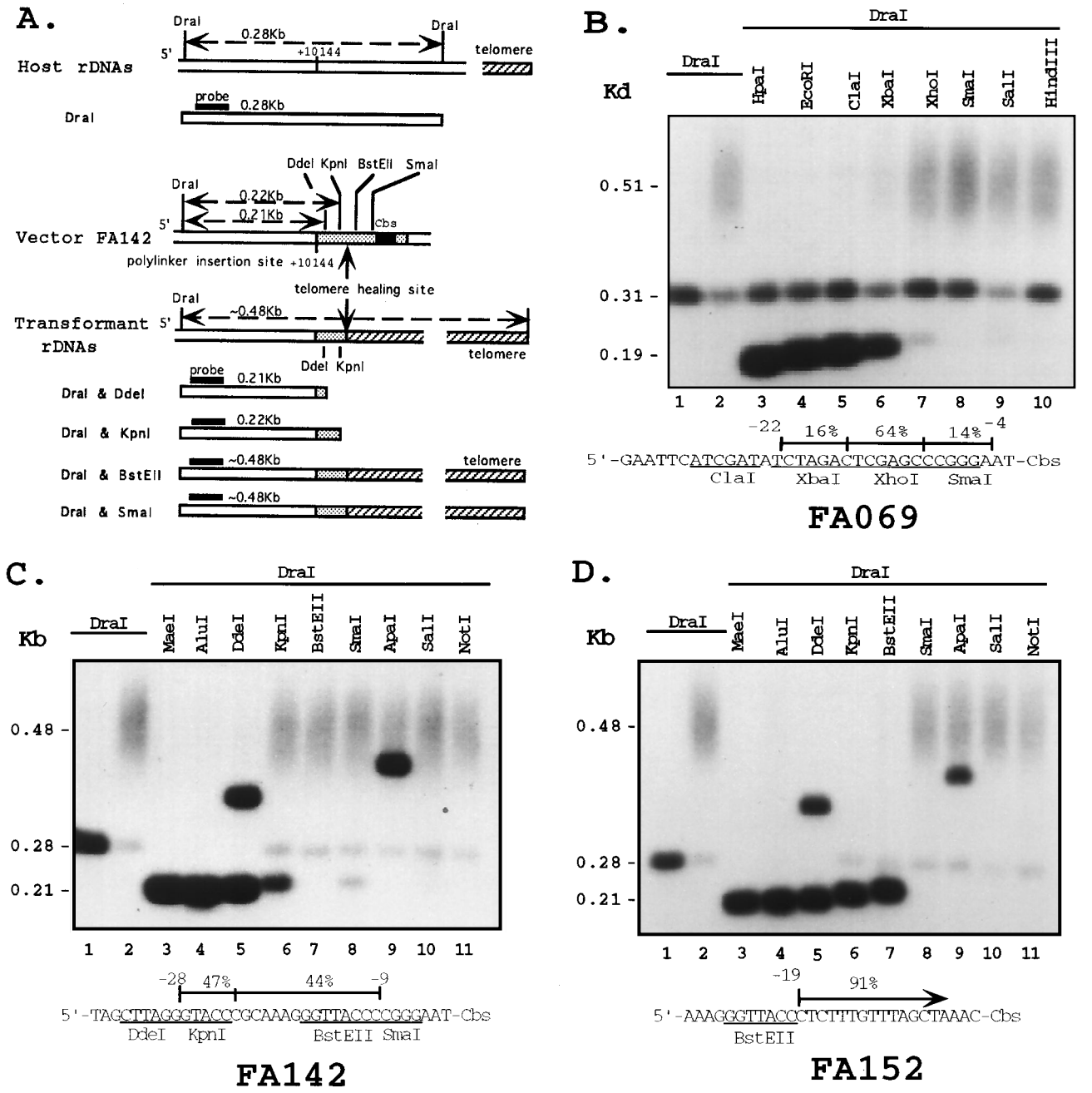

FIG. 2. Restriction mapping of telomere addition sites. (A) Mapping strategy. (B through D) Results of the restriction digestion studies for mapping the telomere addition sites. For simplicity, clone FA142 alone is used as an example in panel A, and only the region of the DNA involved in this analysis is shown. The solid bars indicate the regions complementary to the oligonucleotide sequence used as a probe. The shaded areas represent the linker region, and the hatched bar represents the added telomeric DNA. Only four restriction sites in the inserted linker region are illustrated. The *Dra*I fragment sizes of the host rDNA and the injected DNA are given above their respective maps. One example of the transformant rDNA is shown, in which telomere addition occurs in the region between the *Bst*EII and *Kpn*I sites. Double digestion with *Dra*I and *Dde*I or *Kpn*I generates a 0.21- or 0.22-kb fragment, and double digestion with *Dra*I and *Bst*EII or *Sma*I gives a fragment of approximately 0.48 kb in size. This fragment includes telomeric DNA and is variable in size. (B, C, and D) Restriction digestion results of cells transformed with clones FA069, FA142, and FA152, respectively. Equal amounts of DNA from four (B), eight (C), or six (D) transformants were mixed. Approximately 3 μg of each of these mixtures was digested with restriction enzymes and analyzed by agarose gel electrophoresis and Southern hybridization with the oligonucleotide rdn9757 (B) or rdn10026 (C and D) as a probe. Lane 1 contains host DNA from strain CU427 as a control. The other lanes contain transformant DNA. The restriction enzymes used for each digestion (either single or double digestion) are indicated above the lanes. The numbers to the left of the panels are estimated sizes of fragments migrating at these positions. The 0.31-kb (or 0.28-kb) bands are from host rDNA, which also are present in the transformants. The broad bands in the 0.51-kb (or 0.48-kb) region contain terminal fragments of the transforming rDNA. If the rDNA retains the site for the second restriction enzyme used in the double digestion, bands are produced in the 0.19-kb (or 0.21-kb) region. The relative intensities of material in the 0.51-kb (or 0.48-kb) and the 0.19-kb (or 0.21-kb) regions were measured with a PhosphorImager and used to calculate the fraction of DNAs with the given restriction sites. These values were used to calculate the percentages of rDNA molecules terminating within these restriction sites, which are shown in the lower half of the figure, together with the relevant nucleotide sequence in this region. A small amount of 0.21-kb DNA was detected in lane 8 (*Sma*I digestion) but not lane 7 (*Bst*EII digestion) in panel C. Our interpretation is that a small number of ends are formed by adding GGGTT repeats to the CCCG sequences to the right of the *Kpn*I site, thus creating the unexpected *Sma*I site (CCCGGG). The prominent bands between 0.28 and 0.48 kb in size in lanes 5 and 9 of panels C and D were derived from control plasmid DNA, which was added to these samples to assess the completeness of restriction digestion. The same DNA was also added in other digestions, but the products are not included in this figure because of their large size and slow migration rates.

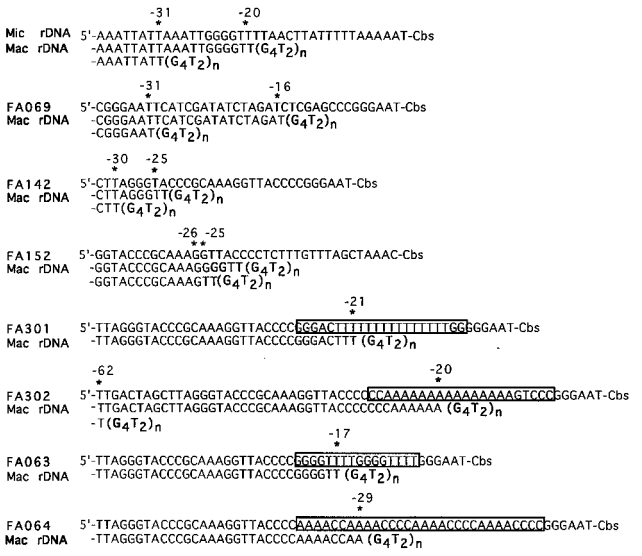

FIG. 3. Nucleotide positions of telomere addition sites. The nucleotide sequences of the transforming rDNAs around the points of telomere addition are shown together with the sequences from the corresponding regions of the injected DNAs (top line of each group). For clones FA301, FA302, FA063, and FA064, the inserted GT- or AC-rich sequences are enclosed in boxes. Different lines in each group represent the sequences from independent transformants. The first group contains the sequence from the normal 3′ end of the rDNA for comparison (20, 25a). The nucleotide positions at which new telomere addition occurs are indicated by asterisks above the sequence of the injected DNA. The numbers above them indicate their distances from Cbs. Mic, micronucleus; Mac, macronucleus.

nucleotide of the injected sequence or the first nucleotide of the added telomeric sequence (Fig. 3). This bias suggested a strong preference for T or G at the addition site, which could be related to the fact that telomerase, which is responsible for this addition (40), has a strong preference for telomere-like sequences as primers in vitro (6, 15, 18, 22).

Telomere additions at CA sequences. The bias toward a G or T raised the interesting possibility that telomerase plays a decisive role in determining the telomere addition site. For instance, DNA cleavage could occur at or near Cbs, and the free ends were degraded by a nuclease until a G or a T appeared at the end before telomere addition could occur. In this case, telomere addition sites would be affected by the presence of T or G in the region surrounding Cbs. To test this idea, we constructed derivatives of FA142 by inserting either of two CA-rich oligonucleotides into the *Sma*I site adjacent to Cbs (Fig. 3). Clone FA302 contained the sequence CCC $(A)_{15}$GTCCC, and clone FA064 contained the sequence AAAACC$(AAAACCCC)_3$ as an insert, which occupied the regions 7 to 28 and 7 to 36 bp from Cbs, respectively. For comparison, two clones (FA301 and FA063) containing similar oligonucleotides inserted in the opposite orientation (thus giving GT-rich sequences in this region) were also constructed and analyzed. For an unknown reason, two of these clones transformed at notably reduced rates (0.4 and 0.3% for clones FA063 and FA064, respectively). Nonetheless, sufficient numbers of transformants were obtained. Their DNAs were subjected to restriction mapping analysis by the method described previously (Fig. 4). In virtually all termini analyzed, the restriction sites to the left of the inserts were retained, indicating that telomere addition occurred within or to the right of the inserted AC-rich sequence. Clone FA064 contained a *Sma*I site between Cbs and the insert. *Sma*I digestion showed that telomere addition occurred to the left of this site. Thus, in this clone, and probably also in the others, most telomere addition sites occurred within the inserted sequence. This study further strengthens the conclusion that local sequences, even if devoid of G or T, do not significantly affect the distance relationship between Cbs and the site of telomere addition.

We amplified the sequences at the rDNA termini from these transformants by PCR and cloned and sequenced these DNAs (Fig. 3). In the two clones that contained stretches of C and A in the region, telomere addition occurs after an A. Thus, although T or G is strongly preferred, A could also be used when G and T are absent. This result further strengthens the conclusion that the distance from Cbs determines the position of telomere addition. The sequence in between has a very limited influence.

Efficient end healing requires Cbs. *T. thermophila* contains an elevated level of telomerase during macronuclear development (4). It is conceivable that cells at these stages could add new telomeres to any broken DNA end efficiently, and not just to those ends generated by breakage at Cbs sites. We investigated this possibility directly by asking if DNA ends generated by restriction enzyme cutting could also be healed in developing macronuclei. The rDNA clone FANA3 lacked the inserted Cbs but was otherwise identical to FA142 or FA152 (Fig. 1). It was cut with the restriction enzymes *Kpn*I, *Bst*EII, and *Sma*I, which each cut the plasmid DNA once in the polylinker region to produce molecules with 3′-protruding, 5′-protruding, and blunt ends, respectively. If these ends could be healed efficiently in developing nuclei, the rDNAs generated in the transformants should be very similar or identical to that found in FA142 or FA152 transformants. Repeated injection of these cut DNAs failed to produce transformants, suggesting that they were incapable of transforming *T. thermophila*. It should be noted that a similar rDNA clone that had been linearized by cutting within the *Escherichia coli* vector sequence transformed *T. thermophila* as efficiently as the circular form did (35). Thus, DNA conformation alone cannot be responsible for the difference. To confirm that the cut rDNA was not healed, we coinjected the digested DNAs with a small amount of uncut DNA (approximately one quarter of the cut DNA). Transformation occurred at normal rates (1.5, 2.8, and 3.7%) as expected. DNAs from several transformed lines were pooled and analyzed by hybridization and restriction mapping (Fig. 5). If the digested DNAs actually acquired telomeres, the terminal *Dra*I fragments should migrate as broad bands around 0.48 kb, much like the ends generated after breakage at Cbs (Fig. 2). This was not found. In all cases, the only nonhost rDNA fragment detected was the 0.36-kb band expected from the coinjected uncut DNA. We conclude that the cut DNAs are not able to form stable ends near the cut sites. This study indicates that not all free ends are able to acquire telomeres efficiently in developing nuclei of *T. thermophila*. Thus, Cbs-induced breakage must possess special properties to promote efficient chromosome end healing.

DISCUSSION

Chromosome breakage occurs in many ciliated protozoa during somatic nuclear differentiation to generate stable DNA fragments with new telomeres (25). In this study, we have analyzed the pattern of telomere addition at one such breakage site in *T. thermophila*. Using specially constructed vectors that contain synthetic copies of the chromosome breakage sequence signal Cbs, we have demonstrated that telomere addition occurs in a distinct pattern. In one construct tested, essentially all telomere addition sites occur within a region 4 to

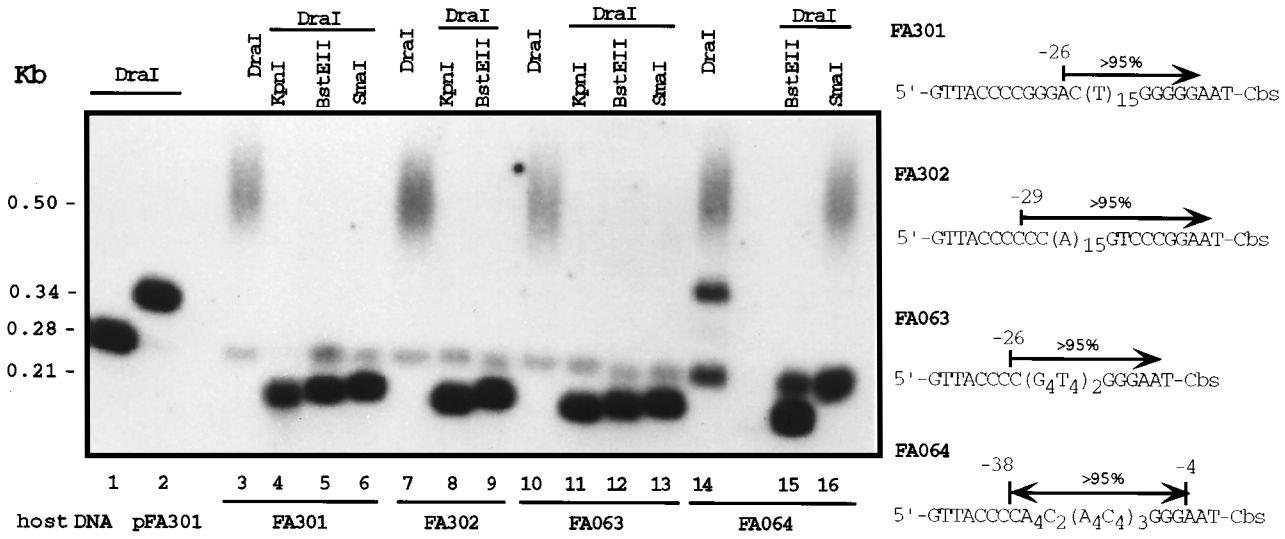

FIG. 4. Restriction mapping of telomere addition sites in regions with biased sequences. DNAs of *T. thermophila* transformants were digested with the restriction enzymes shown above each lane, analyzed by agarose gel electrophoresis and Southern blotting, and hybridized with the oligonucleotide probe rdn10026. DNA clones containing AC-rich or GT-rich inserts were used for transformation. The names of these clones are indicated below the lanes. For each group, a mixture of transformed lines were analyzed: three lines each for FA301, FA302, and FA064 and four lines for FA063. Lane 1 contains host DNA (strain CU427) and lane 2 contains FA301 plasmid DNA as controls. The numbers to the left indicate the estimated sizes of DNA fragments migrating at those positions. The terminal *Dra*I fragments of the transforming rDNAs migrate as broad bands with sizes around 0.50 kb. They are converted to the 0.21-kb band by digestion with the second enzyme if the site is present. The relative intensities of these two bands were measured and used to calculated the fraction of DNA with a given restriction site. This analysis indicates that most (over 95%) of the telomeres are added to the sequences within the inserts, as diagrammed on the right side of the figure. The prominent band between the 0.28- and 0.50-kb bands in lane 14 is derived from control plasmid DNA that had been added to the sample to assess the completeness of restriction digestion.

22 bp from Cbs, 64% of which are within a region 10 to 15 bp from Cbs. In two other constructs in which Cbs is inserted in either orientation and in a different location, 91% of the events occurred between 9 and 28 bp from Cbs in one case and within the first 18 bp in the other. Furthermore, insertion of simple A+C or G+T sequences (8 to 30 bp in length) in the region adjacent to Cbs does not affect this pattern. On the basis of this analysis, it is clear that telomere addition occurs at variable points within a small region that is located a short distance away from Cbs. This basic distribution pattern is not affected by the sequences at the addition sites or the orientation of Cbs.

This pattern of end healing is quite different from that found in other organisms after unregulated chromosome breakage. In the yeast *Saccharomyces cerevisiae*, in which chromosome breakage can be induced through HO endonuclease cutting, end healing occurs at much lower rates at sites hundreds to thousands of nucleotides away, presumably after extensive degradation of the broken ends has occurred (21, 27). Many of these sites contain G+T-rich sequences, and some bear a certain resemblance to telomeric sequences. Furthermore, their occurrence is greatly affected by the presence of a *T. thermophila* telomeric sequence upstream of the breakage site (21). Chromosome end healing associated with other unregulated breakage events may follow a similar pattern (23, 28). The strong preference of telomerase for primers containing telomere-like sequences at or near their 3′ ends in vitro may help explain this phenomenon (6, 15, 18). It is possible that in these cases the broken ends are degraded until internal telomere-like sequences are exposed at or near the ends, which are acted upon by telomerase. The high efficiency of end healing and the close proximity of healing sites to the breakage site in *T. thermophila* represent a very different situation. This apparently is not because these nuclei can heal any broken end efficiently, since restriction enzyme-digested DNAs introduced into these cells were not healed. We thus conclude that telomere addition is tightly linked to the mechanism of chromosome breakage at Cbs sites. This idea is further supported by the fact that sequences devoid of G or T do not prevent telomere addition when placed near Cbs, even though G or T is normally found at these sites.

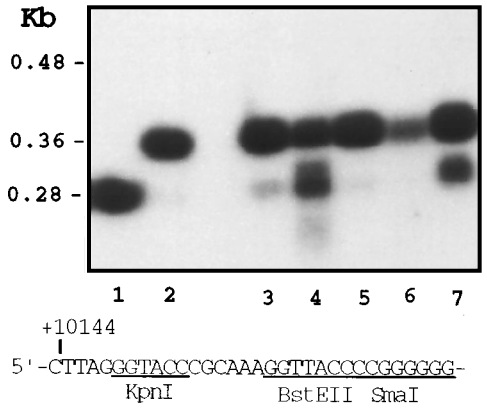

FIG. 5. Telomeres are not formed at ends generated by restriction enzyme digestion. rDNA vector FANA3, which does not contain Cbs in the insert, was cut with a restriction enzyme, mixed with one quarter of the uncut DNA, and injected into mating cells. DNAs from several transformants derived from each injection were mixed and analyzed by *Dra*I digestion, gel electrophoresis, blotting, and hybridization with the oligonucleotide rdn10026 as a probe. Lane 1 contains host DNA, and lane 2 contains FANA3 plasmid DNA. Lanes 3 and 4 each contain mixtures of five different transformants derived from injection with *Sma*I-cut DNA. Lanes 5 and 6 each contain DNAs from four different transformants derived from injection with *Bst*EII-cut DNA. Lane 7 contains a mixture of DNAs from five different transformants derived from injection with *Kpn*I-digested DNA. The estimated sizes of the DNA fragments are indicated on the left side. If the cut DNA was healed by telomere addition, a broad band around 0.48 kb in size would be expected, but this band was not seen in any of the transformants analyzed. The 0.36-kb fragment is the expected fragment from the uncut FANA3 DNA. Thus, the ends produced by restriction digestion are not good substrates for telomere addition in these cells.

The tight association between chromosome breakage and telomere addition in *T. thermophila* offers useful information regarding their underlying mechanisms. Our results indicate that a small but variable DNA segment including Cbs is lost during this process. Because of this loss, the exact location of the initial DNA cleavage has been difficult to determine. It is possible that a single break occurs within Cbs. This break is then followed by a controlled DNA degradation at these ends before telomere addition occurs. Alternatively, two or more breaks may occur on both sides of Cbs at some distance away, thereby deleting the DNA in between. In either case, telomerase must act on these free ends very efficiently after DNA cleavage has occurred. It is perhaps noteworthy that no telomere addition site has been detected within 4 bp of Cbs in our constructs, even though a telomeric sequence (GGGGTT) has been placed in this position to favor telomere formation. In fact, no telomere addition has ever been found within such a short distance of any native or artificial Cbs analyzed so far (36, 37, 40). Thus, it is less likely that the initial cleavage occurs within Cbs. We envision a process in which a protein DNA complex is formed at the Cbs site, which in turn recruits telomerase to the adjoining DNA. DNA cleavage occurs on both sides of Cbs, thus deleting the DNA in between and producing two free ends which are quickly healed by the associated telomerases. In this scheme Cbs is the only *cis*-acting sequence required. Cutting is coupled to end healing through complex formation at Cbs sites. The heterogeneity of the addition sites and their strong bias toward G or T can be explained if the associated telomerase is able to slide slightly along the DNA after breakage and can act on its preferred site within the immediate vicinity after limited DNA degradation has occurred. The presence of nuclease activity in purified *Tetrahymena* telomerase (9) provides one possible explanation for this limited degradation.

By placing sequences containing only A or C next to Cbs, we have created a situation in which end healing occurs at nucleotides other than G or T. These results demonstrate that *Tetrahymena* telomerase is able to act on these nucleotides in vivo. Unlike addition at sites ending in G or T (6, 15, 22), these sites do not contain any nucleotide that is complementary to the telomerase RNA template to influence the addition of the first nucleotide. They provide a special setting for examining the utilization of the telomerase RNA template region, as well as the role of other telomerase RNA sequences on primer selection. It is interesting that in both cases, the telomeric sequences start with 5′-GGGGTT and not any of the other five permutations. This finding suggests that without a telomeric primer sequence, telomerase initiates synthesis by copying the 3′-CCCCAA portion of the RNA template, even though the possible full extent of the RNA template is 3′-AACCCCAAC (16, 17), and the actual template region is believed to be 3′-CCCAAC on the basis of in vitro studies (2, 3).

Efficient end healing is a critical feature of programmed chromosome breakage. In all cases studied, the broken ends acquire telomeres efficiently, and the chromosome fragments produced are stable in somatic cells. This is an important process which ensures somatic genome stability. In organisms other than *T. thermophila* very little is known about the identity of the breakage sequence signal or its regulation. Nonetheless, structural analysis so far has revealed significant differences in telomere formation among these systems. In *Ascaris lumbricoides* (24) and *Paramecium tetraurelia* (11), telomere addition occurs at variable sites that span a region several kilobases in length. It is not known if DNA cleavage occurs at highly variable sites or occurs at fixed sites and is followed by extensive but variable DNA degradation. *P. tetraurelia* has the remarkable ability to add telomeres to most linear DNA fragments injected into its macronucleus during vegetative growth (13, 19). Thus, the coupling of telomere addition to chromosome breakage may not be essential. Whether this is also true for *A. lumbricoides* is not clear. On the other hand, telomere addition occurs reproducibly at the same nucleotide position in *Euplotes crassus*, at least for the one breakage site analyzed (5). This and other hypotrichous ciliates have large numbers of chromosome breakage sites and very small macronuclear DNA molecules (2 to 3 kb on average) with little extragenic DNA (12, 25). It is conceivable that telomere addition is more rigidly coupled with the breakage mechanism in these organisms to prevent accidental loss of essential sequences. *T. thermophila* represents a situation somewhere between these two extremes. Our ability to experimentally manipulate this system should help us better understand this mechanism, which could shed light on the evolution of this process and offer additional insights into the regulation of telomere addition in general.

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