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Characterization of Two Telomeric DNA Processing Reactions in *Saccharomyces cerevisiae*

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We have investigated two reactions that occur on telomeric sequences introduced into *Saccharomyces cerevisiae* cells by transformation. The elongation reaction added repeats of the yeast telomeric sequence $C_{1-3}A$ to telomeric sequences at the end of linear DNA molecules. The reaction worked on the *Tetrahymena* telomeric sequence C_4A_2 and also on the simple repeat CA. The reaction was orientation specific: it occurred only when the GT-rich strand ran 5′ to 3′ towards the end of the molecule. Telomere elongation occurred by non-template-directed DNA synthesis rather than any type of recombination with chromosomal telomeres, because $C_{1-3}A$ repeats could be added to unrelated DNA sequences between the CA-rich repeats and the terminus of the transforming DNA. The elongation reaction was very efficient, and we believe that it was responsible for maintaining an average telomere length despite incomplete replication by template-directed DNA polymerase. The resolution reaction processed a head-to-head inverted repeat of telomeric sequences into two new telomeres at a frequency of 10^{-2} per cell division.

Telomeres, the ends of linear eucaryotic chromosomes, are DNA sequences that provide a stable chromosomal terminus. Telomeric DNA must therefore play a role in overcoming two problems. First, DNA polymerases require a primer and synthesize DNA only in the 5′ to 3′ direction, so that the lagging strand at the end of the chromosome cannot be fully replicated (35). Even a very small extent of incomplete replication would cause a continuing decrease in chromosome length. The second problem is the reactivity of DNA ends. Nontelomeric DNA ends produced by ionizing radiation, mechanical breakage, or restriction enzyme digestion are subject to degradation by nucleases and to fusion by ligation (11, 17–19, 23). Irreversible loss of DNA by degradation would eventually remove essential sequences from a chromosome, while the fusion of two telomeres would create dicentric or ring chromosomes. Dicentric chromosomes are rearranged as the result of chromosome breakage in mitosis, while circular chromosomes give rise to dicentric dimeric circles by sister chromatid exchange.

The model of Bateman (1) (Fig. 1a) provided an elegant solution to the problems of telomere replication and reactivity. He proposed that telomeres end in a hairpin loop that connects the two strands of the DNA duplex. Full replication yields an inverted repeat, which is then processed into two new telomeres by the introduction of staggered nicks, followed by strand separation and snap-back of the overhanging single-stranded ends. Telomeric reactivity is limited by the provision of a hairpin terminus. In a modified form of this model, the processing of the inverted repeat occurs by the formation of a cruciform, which is cleaved at its base by enzymes which recognize Holliday junctions (Fig. 1b). We refer to the processing of an inverted telomeric repeat into two new DNA termini as the resolution reaction.

Since this model was proposed, considerable progress has been made in the analysis of telomeric structure. The terminal several hundred base pairs of all known telomeres are composed of simple sequence DNA. In all cases G residues are found only on the strand which runs 5′ to 3′ towards the terminus of the DNA. For historical reasons, telomere sequences are usually described with respect to the complementary C-rich strand. In many species, long arrays of short repeat units are found: CCCCAA in *Tetrahymena* (3) and CCCTAA in trypanosomes (4, 32). *Oxytricha* is unusual in having a shorter stretch of C_4A_4 repeats (15). In *Dictyostelium* ($C_{1-8}T$) (7) and in *S. cerevisiae* ($C_{1-3}A$) (26), the repeat unit is irregular. In *Oxytricha* the telomere structure is precisely defined: there are a fixed number of C_4A_4 repeats, and the end of the molecule is a double-stranded break with a 16-base-pair (bp) 3′ overhang (15). In other organisms the heterogeneity in the number of telomere repeats has hampered attempts to determine the nature of the DNA terminus. In yeast and *Tetrahymena* cells, single-strand interruptions are found within the telomeric repeats on both the CA- and GT-rich strands (26).

How might these telomeric structures be involved in fulfilling the telomeric functions noted above? It has recently become clear that telomeric DNA is subject to modification by a novel DNA-processing activity that results in the addition of new telomeric sequence to the end of the DNA duplex. It is likely that this elongation reaction plays a central role in telomere function, since all telomeres appear to be subject to the telomere elongation reaction. During the processing of the micronuclear DNA of *Tetrahymena* and *Oxytricha* spp. into macronuclear fragments, the C_4A_2 or C_4A_4 repeats that are added to the ends of these fragments are synthesized de novo (5, 13). Both *Tetrahymena* and *Oxytricha* ends elongate when functioning as telomeres in yeast cells (25, 30, 33). In trypanosomes, the elongation reaction is particularly synchronous, and regular growth of telomeres can be observed (2). While most workers proposed a recombinational mechanism for the elongation reaction (2, 34), the addition of irregular yeast repeats to the regular *Tetrahymena* repeats in yeast cells led Shampay et al. (26) to propose a mechanism involving non-template-directed DNA synthesis (Fig. 1c). Recently, Grieder and Blackburn (8) have shown that during macronuclear development, *Tetrahymena* contains a terminal transferase activ-

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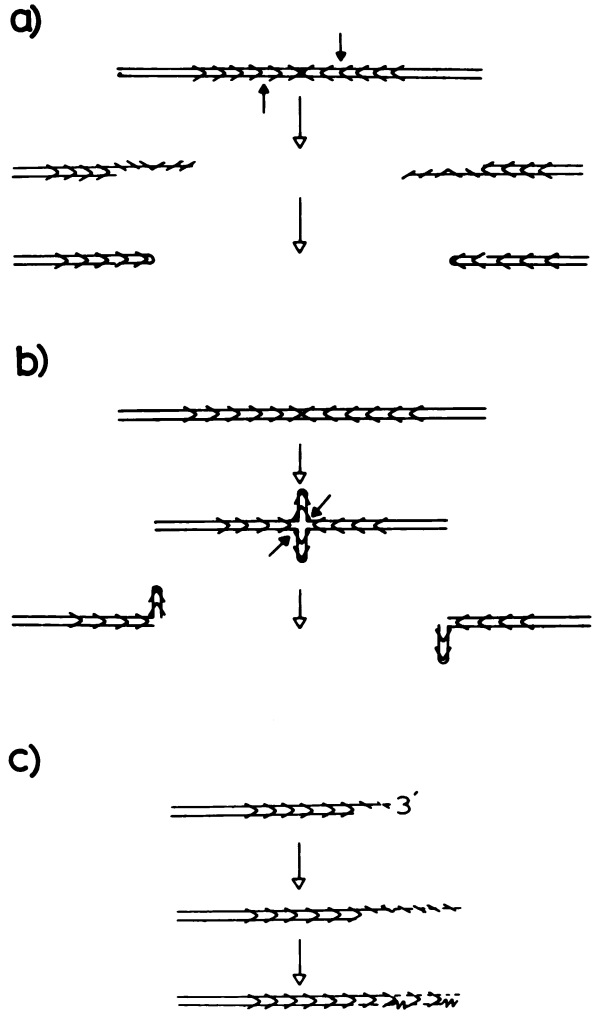

FIG. 1. Models for telomere replication. (a) Bateman model (1). The telomeres are sealed by hairpin loops. Replication around these loops generates an inverted repeat of the telomere. Two nicks (→) are introduced on opposite strands, the region between the nicks is melted to allow the two halves of the repeat to separate from each other, and the single-stranded regions then fold back to recreate the hairpin chromosome terminus. Carets (>, <) indicate location and orientation of the telomeric repeats. (b) Modified Bateman model (29). Replication as in panel a, but resolution via cruciform intermediate. The cruciform is nicked by enzymes which cleave Holliday junctions, to regenerate hairpin termini. (c) Non-template-directed DNA synthesis model (26). A single telomere is shown with a 3′ overhang of the type that would be generated by incomplete telomere replication. This overhang is extended by non-template-directed DNA synthesis. Symbols: – – –, newly replicated DNA; ∿∿, RNA primer for template-directed DNA polymerase.

ity that can add T_2G_4 repeats to single-stranded T_2G_4 or TG_{1-3} primers (which correspond to the sequences which run 5′ to 3′ towards the termini of *Tetrahymena* and yeast telomeres, respectively) in the absence of any template. This enzyme has an essential RNA component; it is possible that the RNA component determines the sequence of the DNA that is recognized and/or synthesized (9).

We have investigated the telomere resolution and elongation reactions in yeast by introducing DNA molecules of defined structure into yeast cells and then following their fate in vivo. In particular we have investigated the substrate specificities of these reactions and attempted to determine which of them is responsible for the completion of telomere replication. The efficiency of the elongation reaction suggests that it plays a central role in telomere replication. Our experiments support the involvement of non-template-directed DNA synthesis in the elongation reaction and argue against a recombinational mechanism.

MATERIALS AND METHODS

Enzymes, chemicals, and media. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and used as recommended by the manufacturer. Medium components were from Difco and Sigma, and media were prepared as described before (27).

Strains. The bacterial strains JA300 (31) and BA1 (*leuB6 trpC1116 hisB thyA thi* Str^r Tc^r *hsdR hsdM*; A. W. M., unpublished) were used for plasmid constructions. Bacterial plasmids used in this study were constructed by standard recombinant DNA techniques. The ARS5 fragment used in the construction of pTC26 is from the yeast *SUP4* region and was the gift of R. Rothstein (unpublished). The yeast strains used for transformation were T1753 (α *leu2-3,112 his3-11,15 ura3 trp1 can1 cir*^{0}) and D234.3B (α *leu2-3,112 his3-11,15 ura3 trp1 can1 tcm1 cir*$^+$). Some transformants were mated to strains DM64.2A (**a** *leu2-3,112 his3-11,15 ura3 arg4 cir*$^+$) or DM64.5B (**a** *leu2-3,112 his3-11,15 ura3 arg4 cir*$^+$).

Yeast transformation and Southern blots. Yeast spheroplast transformations and Southern blots (28) were performed as described by Orr-Weaver et al. (24). In some cases, LiCl-mediated transformation (12) was used.

Creation of a chromosomal inverted repeat. The plasmid A193p10 (Fig. 2) contains an inverted repeat of the *Tetrahymena* telomeres (referred to as Tr ends) separated by the *HIS3* gene, a second copy of *HIS3*, and a 3.7-kilobase (kb) fragment containing the *MAT***a** locus. The plasmid was first cleaved with *Bam*HI and then recircularized with DNA ligase to create an uninterrupted repeat of the Tr ends. The ligated DNA was then cut with *Xba*I, which cuts in the *MAT***a** DNA, targeting the plasmid to recombine with the *MAT* locus when it is introduced into a yeast cell by transformation (24). The *Xba*I-cut DNA was introduced into strain D234.3B, and His$^+$ transformants were selected and then screened to identify those in which the integrated *HIS3* gene was mitotically stable. DNA from 12 of these transformants was prepared and examined to identify transformants with the expected structure. We found two transformants, T2176 and T2181, with the correct structure. Because DA234.3B is *MAT*α and the integrated plasmid carries *MAT***a**, these transformants are *MAT***a**/*MAT*α haploids which mate extremely poorly. Rare diploid derivatives were selected after mating T2176 or T2181 to the tester strains DM64.2A and DM64.5B. We examined DNA from 28 diploids and identified 9 which had maintained the inverted repeat of the Tr ends flanked by duplications of *MAT* DNA. Four of these strains, DA309, DA335, DA336, and DA354, were used for pedigree analysis to determine the frequency of telomere resolution as described below.

Resolution frequency measurements. The frequency at which an inverted repeat of the Tr ends integrated at the *MAT* locus of chromosome III resolved was measured by pedigree analysis (22). An exponentially growing culture of a diploid strain which carried one copy of the modified chromosome III was streaked onto a nonselective plate, and buds were separated from mother cells by micromanipulation. This separation was repeated for at least four generations to yield defined cell lineages. The single cells were allowed to

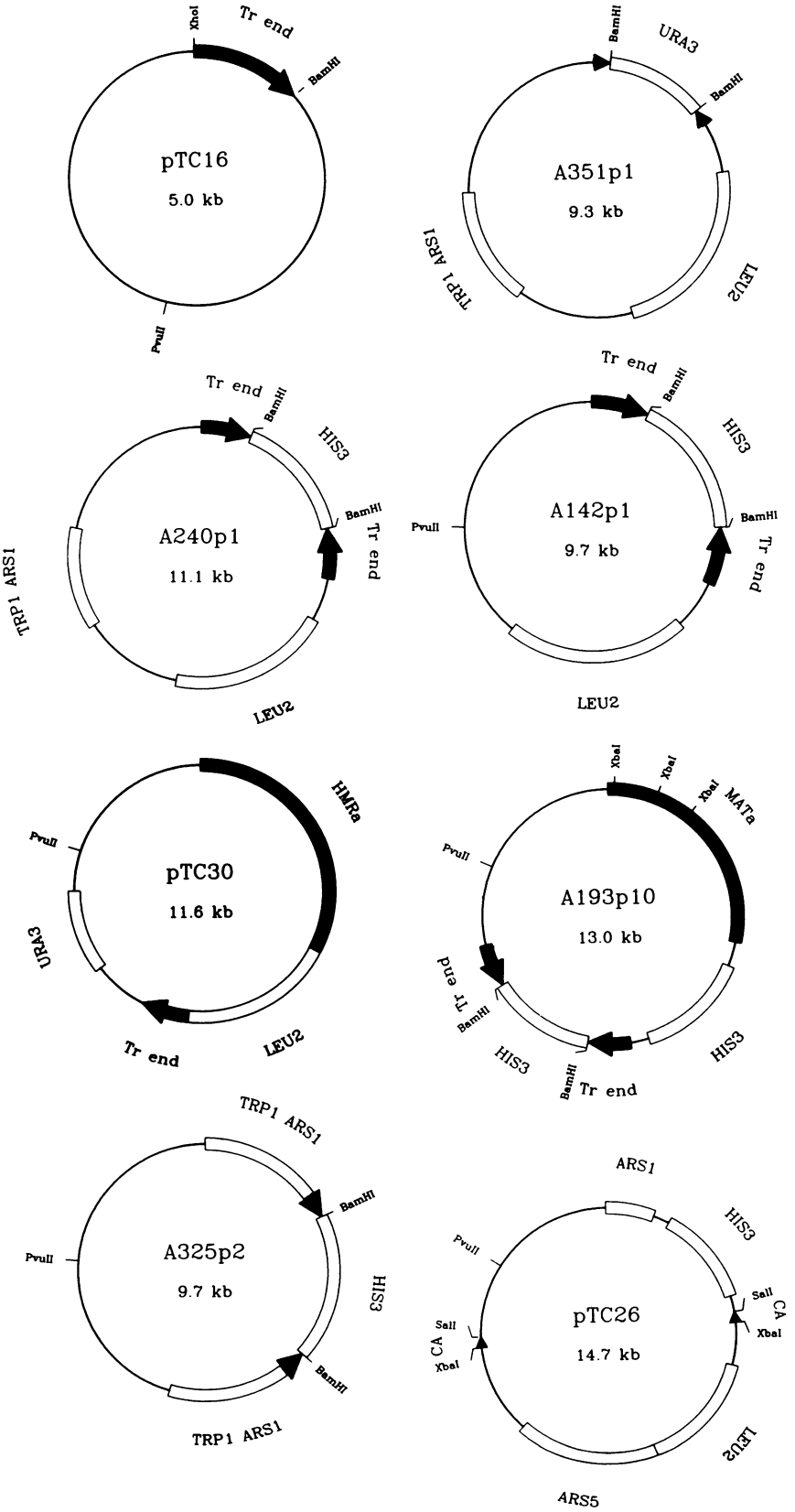

FIG. 2. Plasmid maps. The maps of plasmids used in this paper are shown. All plasmids are shown with the unique *Eco*RI site at twelve o'clock and the ampicillin resistance gene reading counterclockwise from this point. The position and extent of regions of yeast DNA or Tr ends and the location of certain restriction enzyme sites are shown. Solid arrows, Telomeric sequences.

grow into colonies and then replica plated to medium lacking either histidine, leucine, or uracil. Resolution events break chromosome III at *MAT*, creating a centric fragment which carries *HIS3* and an acentric fragment which carries *LEU2* and *URA3* (see Results). The acentric is rapidly lost (20), yielding cells which are His^+ but Leu^- and Ura^-. Cell divisions which yield both His^+ Leu^+ Ura^+ and His^+ Leu^- Ura^- cells are candidates for resolution events. All putative resolution events were verified by preparing DNA from the Leu^- Ura^- strains and demonstrating the presence of a new telomere at *MAT* by Southern blot analysis.

RESULTS

Elongation reaction. When the *Tetrahymena* telomeric repeats (henceforth referred to as Tr ends) are introduced into yeast cells, the elongation reaction adds 100 to 300 bp of the yeast telomeric $C_{1-3}A$ repeat to their ends (26, 30, 33). We investigated the efficiency and substrate specificity of the elongation reaction. We tested whether Tr end fragments ending in a simple restriction enzyme cut were efficient substrates for the elongation reaction. The plasmid A240p1 (Fig. 2) contains the yeast *LEU2* gene as a selectable marker and the *ARS1* replicator as well as an interrupted, head-to-head Tr end repeat in which the Tr ends are separated from each other by the yeast *HIS3* gene. The separation of the Tr end sequences from each other allows the plasmid to be propagated in *Escherichia coli* (plasmids that carry an uninterrupted inverted repeat are rapidly rearranged [16]). When this plasmid is cut with *Bam*HI, the *HIS3* gene is removed, leaving a linear fragment that terminates with Tr end sequences, with 12 bp of pBR322 DNA at the extreme terminus. When this DNA was introduced into yeast cells by transformation, it gave rise to linear plasmids of the expected structure, whose telomeres were subjected to the elongation reaction (Fig. 3a). On the basis of previous experiments (26), we assume that the added DNA consists of $C_{1-3}A$ repeats. To test the efficiency of this reaction, we compared the number of transformants yielded by uncut A240p1 DNA with that yielded by *Bam*HI-cut DNA. The *Bam*HI-cut and uncut DNA gave rise to 9,700 and 11,000 transformants per μg of DNA, respectively. The high transformation frequency with linear DNA which terminated in Tr end sequences was in marked contrast to the dramatic reduction in transformation when plasmids were cut within pBR322 sequences (23) and suggests that almost all the molecules ending in Tr ends introduced into a cell are capable of being converted into telomere-bearing linear plasmids. Thus, the C_4A_2 telomeric repeat of *Tetrahymena* is an excellent substrate for the elongation reaction.

The Tr end fragment on A240p1 is the 700-bp terminal *Hin*dIII fragment of the linear extrachromosomal *Tetrahymena* rDNA. The 700-bp *Tetrahymena* fragment has been fully sequenced (14). It contains approximately 400 bp of unique AT-rich DNA followed by 300 bp of C_4A_2 repeats at the end corresponding to the terminus of the linear rDNA. The AT-rich DNA contains a sequence that can act as a weak ARS element (origin of replication) in yeast cells. We wished to know whether this unique DNA played any role in the ability of this fragment to act as a telomere in yeast or whether the C_4A_2 repeats alone were sufficient for full telomere function.

The generation of deletions in this fragment, and their subsequent analysis, was facilitated by recloning the 700-bp fragment from pSZ221 (30). The resulting plasmid, pTC16 (Fig. 2), has an *Xho*I site adjacent to the unique DNA and a

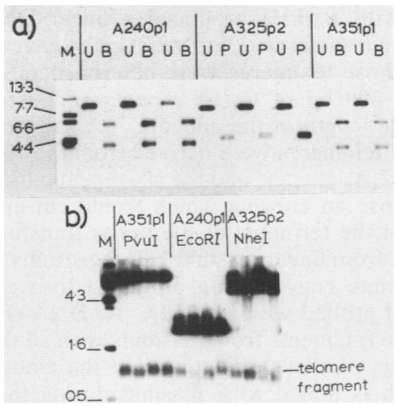

FIG. 3. Linear plasmids with differing amounts of *Tetrahymena* DNA at their termini. (a) Southern blot of linear yeast plasmids probed with pBR322. The plasmids were derived by transforming strain TA1753 with *Bam*HI-cut DNA from plasmids A240p1 (300 bp of C_4A_2 and 350 bp of unique-sequence *Tetrahymena* DNA), A325p2 (300 bp of C_4A_2 DNA), and A351p1 (36 bp of C_4A_2 DNA). The blot shows three transformants derived from A240p1, three from A325p2, and two from A351p1. DNA from each transformant was run uncut (U) or cut with *Bgl*II (B) or *Pvu*II (P), which cut only once within the plasmid. The production of two restriction fragments by enzymes which cut a plasmid once is diagnostic of linear plasmids. The two fragments produced from the linear plasmid derived from A325p2 comigrate. Lane M, Size standards whose length (in kilobases) is shown in the left margin. (b) Measuring the length of telomeric fragments from linear plasmids. DNA from four transformants derived from each of the plasmids A351p1, A240p1, and A325p2 was cut with the indicated restriction enzymes, run on a 0.7% agarose gel, blotted, and probed with pTC16. Lane M contains standards whose size is shown in the left margin. The distance from the indicated restriction site to the terminus of the transforming DNA is 685 bp for A240p1, 715 bp for A325p2, and 690 bp for A351p1.

*Bam*HI site at the end of the cluster of C_4A_2 repeats. The *Bam*HI site corresponds to the end of the *Tetrahymena* rDNA molecule. Deletions were made from the *Xho*I site through the unique DNA towards the telomere repeats with the exonuclease *Bal*31, and two cloned deletion fragments were sequenced. One fragment had lost all of the unique AT-rich DNA but little else: it consisted of 50 C_4A_2 repeats (in pTC5) flanked by linkers. The second fragment consisted of only six C_4A_2 repeats (in pTC2), also flanked by linkers.

These fragments were assayed for their ability to act as substrates for the telomere elongation reaction. The 50-repeat cluster from pTC5 was used in the construction of a circular plasmid, A325p2 (Fig. 2), that contained the yeast *HIS3* gene flanked by C_4A_2 clusters in inverted orientation. Digestion of this plasmid with *Bam*HI generated linear DNA molecules with C_4A_2 clusters at each end. This DNA was able to transform yeast with high frequency, and all of the transformants carried linear plasmids (Fig. 3a). The telomeres of these plasmids had been modified by the elongation reaction. The original homogeneous end fragments had become longer by 200 to 300 bp, and heterogeneous in size with a dispersion of 100 to 200 bp (Fig. 3b). In previous experiments, the DNA added to C_4A_2 repeats in yeast cells was shown to be yeast $C_{1-3}A$ repeats (26). Thus, the unique DNA adjacent to the C_4A_2 repeats is not necessary for telomere function in yeast cells.

The six-repeat cluster was assayed for telomere function in a similar way. The plasmid A351p1 contains two inverted copies of the six-repeat cluster separated by the *URA3* gene.

Digestion with *Bam*HI produced a linear DNA fragment which, when transformed into yeast cells, gave rise to linear plasmids whose telomeres were heterogeneous in size and had 200 to 300 bp of $C_{1-3}A$ repeats added to them. We analyzed the length of the added $C_{1-3}A$ sequences on plasmids whose telomeres were derived from the 700-bp Tr end, the 300-bp C_4A_2 cluster, and the six-repeat cluster. For each case we chose an enzyme which would cut approximately 700 bp from the terminus of the linear transforming DNA. Yeast DNA from linear plasmid-bearing strains was cut with the appropriate enzyme, run on an agarose gel, Southern blotted, and probed with labeled C_4A_2 DNA (Fig. 3b). The length of the fragments from plasmids with all three types of end was very similar, indicating that the amount of $C_{1-3}A$ DNA which is added to a plasmid during the elongation reaction is not influenced by either the unique *Tetrahymena* DNA or the amount of C_4A_2 DNA at the end. Thus, any system which monitors and regulates the amount of $C_{1-3}A$ DNA at telomeres does not detect C_4A_2 DNA as bona fide telomeric DNA. C_4A_2 repeats cannot be the normal substrates for the resolution and elongation systems, since there are no such sequences in the yeast genome. The most likely normal substrates are the stretches of $C_{1-3}A$ found at yeast telomeres (26); cloned $C_{1-3}A$ clusters are indeed substrates for the reaction (V. Lundblad and J. W. Szostak, unpublished).

Since yeast telomeres contain short regions of alternating CA within the $C_{1-3}A$ clusters, we tested a fragment of pure alternating CA for substrate activity. A 150-bp fragment consisting of $(CA)_{75}$ flanked by linkers [from pHU(TG)$_n$-5, a gift of D. Treco] was used in the construction of pTC26 (Fig. 2), in which two CA clusters were arranged as an inverted repeat separated by 7.3 kb of DNA. Digestion of pTC26 with *Sal*I generated linear molecules carrying *ARS5* (the gift of R. Rothstein) and the *LEU2* gene, which had CA clusters at both ends with the GT-rich strand running 5′ to 3′ towards the telomere as it would in a normal yeast telomere. These molecules were gel purified and used to transform yeast cells. The transformants carried linear plasmids, and the telomeres of these plasmids had been modified by the elongation reaction (Fig. 4d). Thus, alternating CA is also a substrate for the elongation reaction.

Orientation specificity of the elongation reaction. All telomeric sequences have the same orientation: the GT-rich strand runs 5′ to 3′ towards the DNA terminus, and the CA-rich strand runs away from it (3, 4, 7, 15, 26, 32). In the experiments described above, all of the CA-rich clusters had this orientation in the linear molecules that were used to transform yeast cells. We tested the orientation specificity of the elongation reaction by transforming yeast cells with linear molecules containing terminal CA clusters oriented so that the CA-rich strand ran 5′ to 3′ towards the DNA terminus. Digestion of pTC26 (Fig. 2) with *Xba*I resulted in the formation of linear molecules carrying *ARS1* and the *HIS3* gene which were terminated by alternating CA clusters with this "incorrect" orientation. This fragment transformed yeast cells with reduced efficiency, and none of the transformants contained linear plasmids. Restriction analysis of the plasmids that were recovered revealed circular plasmids containing a variety of deletions and rearrangements (data not shown). Thus, the alternating CA cluster is a substrate for the elongation reaction only when the CA-rich strand runs in the same direction as it does in a natural telomere.

Location of sequences recognized by the elongation system. Sequence analysis of two *Tetrahymena* ends modified in yeast showed that the yeast repeats were added directly to

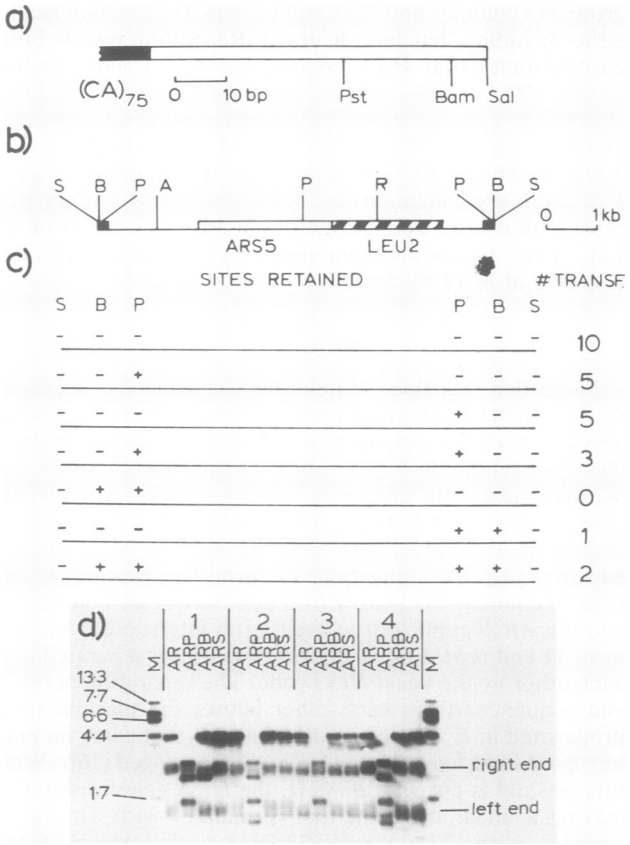

FIG. 4. Retention of nontelomeric DNA at the ends of linear plasmids. (a) Map of the terminus of the *Sal*I fragment of pTC26 used to transform yeast. Solid bar, CA DNA. (b) Map of the *Sal*I fragment of pTC26 used to transform yeast cells. Symbols for restriction sites: S, *Sal*I; B, *Bam*HI; P, *Pst*I; A, *Ava*I; R, *Eco*RI. (c) Schematic of restriction sites retained in transformants derived from pTC26. Each line indicates a particular configuration of retained restriction sites and the number of transformants with this structure. (d) DNA from four transformants derived from *Sal*I-cut DNA was cut with the indicated combinations of restriction enzymes, run on a 0.7% agarose gel, blotted, and probed with pBR322 plus *LEU2* DNA. Lane M contains size standards whose sizes are listed in the left margin, while the positions of the telomeric bands are indicated in the right margin. The presence of a restriction site in a telomeric fragment is visible both as a decrease in the size of the fragment in the triple digest that includes the appropriate enzyme and as the disappearance of length heterogeneity in this band. Transformant 1 has both *Pst*I and *Bam*HI sites in the right telomere, transformant 2 has *Pst*I sites in both telomeres, transformant 3 retains none of the telomeric sites, and transformant 4 retains *Pst*I sites at both telomeres.

the C_4A_2 repeats (6, 26). We were therefore surprised to find that, in some cases, extraneous DNA sequences (e.g., vector and linker DNA) could be retained between the original C_4A_2 or CA repeats and the newly added yeast sequences. Presumably the enzymes involved in telomere elongation recognize sequences that are somewhat removed from the end of the molecule.

We examined the retention of non-CA DNA between the CA cluster and the end of the linear molecule as follows. The circular plasmid pTC26 contains a separated inverted repeat of $(CA)_{75}$ clusters (Fig. 2). Each CA cluster is inserted within a polylinker sequence, so that they are followed by the restriction enzyme sites *Pst*I, *Bam*HI, and *Sal*I (Fig. 4a). The distance from the end of the CA cluster to the *Pst*I,

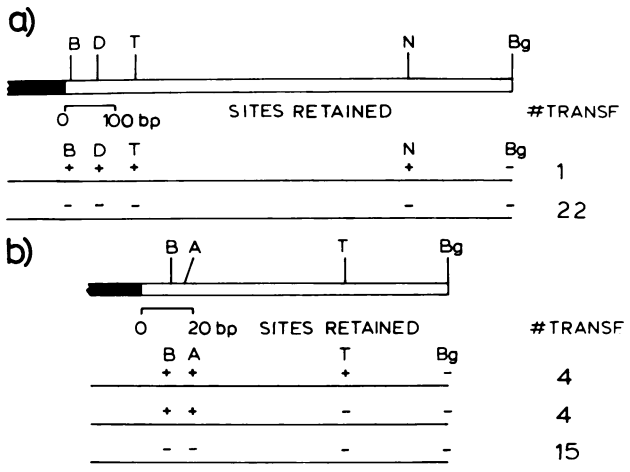

FIG. 5. Retention of large regions of telomeric DNA. Plasmids were produced by transforming strain T1753 with *Bgl*II-cut DNA from plasmid A142p1 and then analyzed for the retention of nontelomeric DNA sequences as in Fig. 4. (a) Retention of DNA at the long end of the transforming DNA. Map of the terminus which carries 800 bp of nontelomeric DNA. Underneath are the two different patterns of site retention observed. The single transformant which retained at least 600 bp of nontelomeric DNA did not retain any of the telomeric restriction sites on its other telomere. Restriction site symbols: B, *Bam*HI; D, *Dra*I; T, *Taq*I; N, *Nde*I; Bg, *Bgl*II. Solid bar, C_4A_2 DNA. (b) Retention of DNA at the short end of the transforming DNA. The map of the DNA as it enters the cell and the numbers of transformants with different configurations of retained restriction sites are shown.

*Bam*HI, and *Sal*I sites is 33, 59, and 66 bp, respectively. *Sal*I-cut DNA was used to transform yeast cells, and 26 linear plasmids were examined for retention of the *Sal*I, *Pst*I, or *Bam*HI sites between the CA cluster and the added $C_{1-3}A$ DNA (Fig. 4d). The results of this analysis are summarized in Fig. 4c. Ten of the plasmids retained none of the restriction sites at either end of the molecule. Ten plasmids retained the *Pst*I site at only one end of the molecule, and three retained it at both ends. One plasmid retained both the *Bam*HI and *Pst*I sites at only one end, and two retained these sites at both ends. None retained the *Sal*I site at either end. Overall, 33% of the *Pst*I sites were retained, showing that the yeast telomeric repeats are often added to the introduced molecules when there are at least 35 bp of DNA between the CA cluster and the end of the molecule.

In the above experiments, as much as 60 bp of DNA was retained between the preexisting CA cluster and the newly added $C_{1-3}A$ DNA. We wished to know whether larger amounts of DNA could be retained or whether there was some limit to the length of the intervening non-CA DNA. The plasmid A142p1 contains an inverted repeat of the Tr ends separated by the *HIS3* gene (Fig. 2). We cut A142p1 within the *HIS3* gene with *Bgl*II to generate linear molecules terminated by C_4A_2 clusters followed by 120 bp of unrelated DNA at one end and by 870 bp of unrelated DNA at the other end. The restriction sites available for the determination of the extent of the retained DNA are shown in Fig. 5. *Bgl*II-cut A142p1 DNA was used to transform yeast cells, and we examined the structure of 23 linear plasmids. None of the plasmids retained the terminal *Bgl*II sites. At the long end, one of the transformants retained at least 670 bp of DNA between the C_4A_2 repeats and the $C_{1-3}A$ DNA added by the elongation reaction. None of the remaining transformants retained any of the sites at this end. At the short end, eight plasmids showed retention of the *Ava*I and *Bam*HI sites, which were 17 and 12 bp beyond the end of the C_4A_2 repeats, respectively, and five of these also retained a *Taq*I site which was 80 bp beyond the end of the C_4A_2 repeats. In all other cases, the unrelated DNA was apparently degraded before the elongation reaction started. We conclude that the elongation reaction rarely retains more than 100 bp of nontelomeric sequence between the introduced telomeric sequences and the added $C_{1-3}A$ DNA.

Resolution reaction. We have previously shown that an inverted repeat of the terminal 700 bp of the *Tetrahymena* rDNA plasmid is resolved in yeast cells to yield two functional telomeres (29). We have constructed linear DNA molecules that terminate in Tr ends and whose extreme termini are hairpin loops; these molecules, when introduced into yeast cells by transformation, also give rise to linear plasmids. The telomeres on these plasmids and also on those derived from molecules which contained head-to-head Tr end repeats have become longer by the addition of 100 to 300 bp of DNA. Thus, two of the intermediates postulated by the Bateman (1) model can undergo a resolution reaction and give rise to telomeres which are subsequently substrates for the elongation reaction. Neither inverted repeats nor hairpin termini consisting of nontelomeric sequences are capable of being converted into telomeres (29).

We have examined the resolution of two new inverted repeat structures by transformation of yeast cells with circular plasmids. An inverted repeat of alternating CA repeats was made by *Bam*HI digestion of a plasmid (pTC27) that has CA clusters flanking *HIS3*, followed by ligation and gel purification of supercoiled circles. Examination of DNA from six transformants revealed only linear plasmids. In another experiment, a plasmid (pTC7) was constructed with a pseudo-inverted repeat composed of C_4A_2 repeats on one side and CA repeats on the other. This plasmid (which was stable in *E. coli*) was used to transform yeast cells, and 10 transformants were examined by Southern blot analysis. Two had resolved to yield linear plasmids, while eight had remained circular. The circular plasmids were able to resolve at a low frequency, since linear plasmids could be formed after extensive subculturing.

The rate of the resolution reaction in such experiments is difficult to measure, since many generations elapse between the transformation event and the preparation of DNA for structural analysis. Measurements of the fraction of plasmid molecules that are linear as a function of time after introduction of a head-to-head telomeric repeat will greatly overestimate the rate of resolution, because acentric linear plasmids are more mitotically stable than acentric circular ones (21). To accurately measure the frequency of telomere resolution, we designed a scheme by which resolved and unresolved molecules would be equally well propagated and resolution events would be detectable genetically as well as physically. This scheme involved the introduction of a head-to-head repeat of the Tr ends at the *MAT* locus of chromosome III (Fig. 6a). Resolution of this inverted repeat into two new telomeres breaks chromosome III into two halves. The left half carries all sequences to the left of *MAT*, including the centromere of chromosome III, and is stably propagated. The right half carries all the sequences to the right of *MAT*, and because it lacks a centromere, it is rapidly lost by mitotic segregation (20). In a diploid strain which carries a normal version of chromosome III as well as the version carrying the Tr end repeat, the resolution events are detected by the loss of the *LEU2* and *URA3* genes which

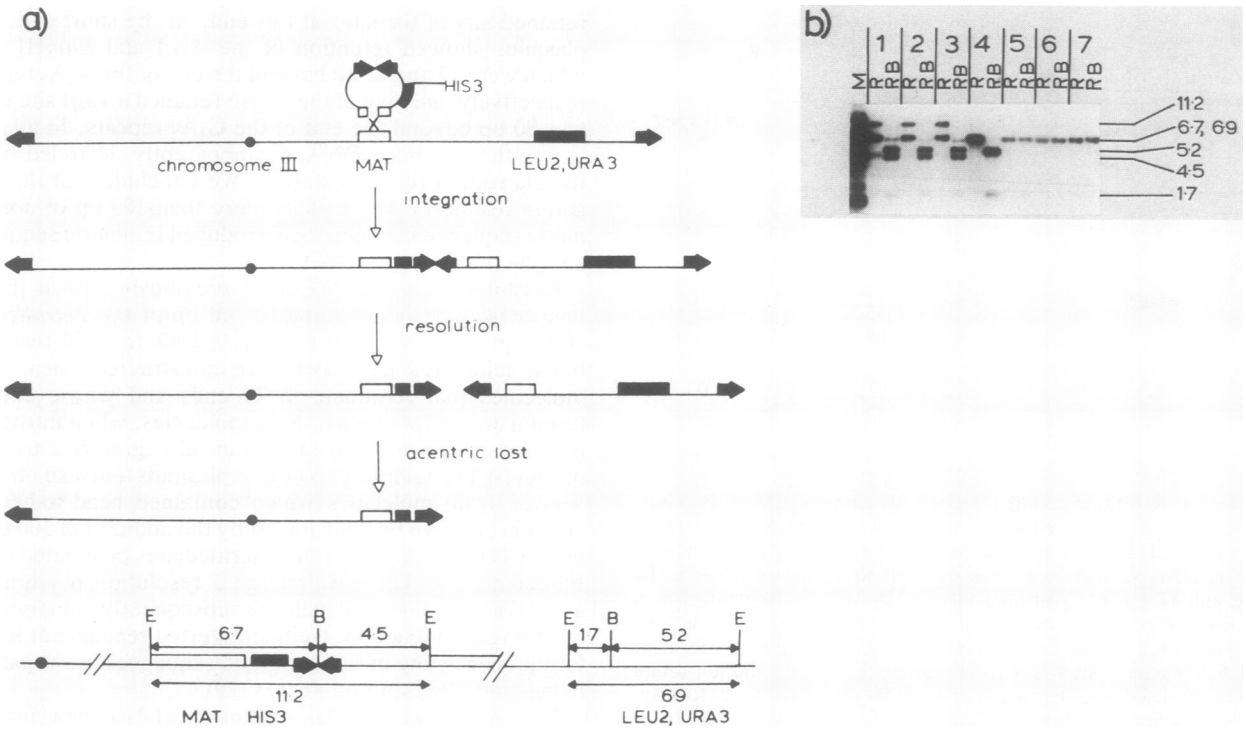

FIG. 6. Creation of an integrated Tr end repeat. (a) Construction and resolution of an inverted repeat of the Tr ends integrated into chromosome III. Symbols: solid circle, centromere; solid arrow, telomeric sequence; open box, *MAT*; solid box, selectable yeast genes. The indicated *LEU2* and *URA3* genes had been previously integrated close to the right telomere of chromosome III. The last line shows sizes of the restriction fragments generated by *Eco*RI (E)-*Bam*HI (B) or *Eco*RI digestion which will hybridize to pBR322 or Tr end DNA. (b) Southern blot of *Eco*RI (R) and *Eco*RI-*Bam*HI (RB) digests of yeast DNA probed with pTC16. DNA was derived from the inverted repeat-containing strains DA309A (lanes 1), DA335A (lanes 2), and DA336A (lanes 3), the control strain T1733 which carries pBR322 sequences at *HMR* but not at *MAT* (lanes 4), and resolved derivatives of the Tr repeat (lanes 5–7). Note that in the resolved derivatives, bands characteristic of the intact inverted repeat (the 11.2-kb *Eco*RI band) and the pBR sequences found at *HMR* (the 5.2-kb *Eco*RI-*Bam*HI band) have disappeared. Lane M contains size standards, and the positions of the bands indicated in Fig. 6a are shown on the right margin.

have been integrated near the right telomere of the chromosome carrying the Tr end repeat. The Tr end repeat was introduced into a haploid strain; we identified two transformants which carried the correct head-to-head Tr end repeat and mated them to a haploid which contained a normal version of chromosome III. The resulting diploid strains were subjected to pedigree analysis, in which defined cell lineages are produced by separating individual mother and daughter cells (22), and then we examined them for inheritance of the *LEU2* and *URA3* genes. Putative resolution events were identified as cell divisions which gave rise to Leu^- Ura^- cells. In all cases their identity was confirmed by preparing DNA from the Leu^- Ura^- strains and demonstrating that a new telomere had been generated at the site of the head-to-head Tr end repeat (Fig. 6b). There were 11 resolution events in 970 cell divisions, giving a resolution frequency of 1.1×10^{-2} per cell division. This frequency is too low to account for the processing of normal yeast chromosomal telomeres if inverted repeats are an obligatory intermediate in telomere replication. Because the substrate for resolution was an inverted repeat of the *Tetrahymena* ends rather than the bona fide yeast telomeric sequences, we cannot rule out the possibility that resolution of an inverted repeat of yeast telomeric sequences would occur at a much higher frequency.

DISCUSSION

We have investigated the rates and substrate specificities of the telomere elongation and resolution reactions in yeast in an attempt to determine the way in which chromosomal telomeres are fully replicated. Earlier experiments involving transformation of yeast with *Tetrahymena* (26) and *Oxytricha* (25) telomeres argued that the completion of telomere replication involves non-template-directed DNA synthesis at a free telomeric 3′ end rather than replication around a hairpin loop followed by resolution of an inverted telomere repeat (1). This paper extends those observations by providing information about the specificity of the reaction that adds yeast $C_{1-3}A$ repeats.

We have shown that the telomere elongation system can recognize C_4A_2 and CA repeats, but only when these sequences are in the correct orientation and when they are present at or close to the end of a linear DNA molecule. Ends from *Oxytricha*, consisting of a short stretch of C_4A_4 repeats, are also elongated in yeast (25), as are trypanosome telomeres, with CCCTAA repeats (4). It should be emphasized that unrelated sequences are not substrates for the elongation reaction. Linear molecules with a variety of unique sequence termini have not yielded linear plasmids when introduced into yeast by transformation (23, 30). The details of the substrate specificity remain to be elucidated, but it is likely that the enzymes involved in the elongation reaction recognize the general feature of C-richness of one strand and G-richness of its complement, either directly or by perturbations in the DNA structure that this sequence composition causes. Another example of apparent general sequence specificity is element II of yeast centromeres: all centromeres have an element II which is more than 95% A:T

base pairs, but the base sequence is not conserved between different centromeres.

Since linear molecules ending in Tr ends are efficiently converted into linear plasmids, the elongation reaction itself must be very efficient. One major advantage of the elongation reaction as a means of solving the problem of complete telomere replication is that, unlike the resolution reaction, it need not occur in every cell cycle. For instance, if incomplete terminal replication causes the loss of 15 bp of DNA per cell generation (in the absence of the elongation reaction), then a telomere which has 300 bp of telomeric repeat sequence will be able to survive more than 10 cell cycles without the elongation reaction occurring. In this model, the structure of telomeres is not static but is in dynamic equilibrium as the result of the opposing actions of non-template-directed DNA synthesis on the one hand and incomplete replication and exonucleolytic degradation on the other. This dynamic equilibrium provides a ready explanation for the observed heterogeneity in the length of yeast and ciliate telomeres. The constant average length of yeast telomeres implies a feedback mechanism which senses the length of telomeric DNA and reduces the extent of non-template-directed DNA synthesis when the telomeric DNA exceeds a certain length. Although the elongation reaction recognizes C_4A_2 repeats, the feedback system is apparently more specific and can distinguish between C_4A_2 repeats and $C_{1-3}A$ repeats. The continuous increase in the length of trypanosome telomeres (2) most likely results because the elongation reaction outstrips degradation. Perhaps this imbalance reflects the absence of a feedback system for controlling the elongation reaction. Proof that the elongation reaction is responsible for normal telomere replication will require the isolation of mutants defective in the elongation pathway.

What is the mechanism of the telomere elongation reaction? Shampay et al. (26) have proposed that the addition of $C_{1-3}A$ repeats to telomeres in yeast cells involves non-template-directed DNA synthesis. Specifically, they proposed that the 3′ end of the GT-rich strand is extended by a sequence-specific terminal transferase-like activity that can add G residues after a T and either G or T after G. The CA-rich strand is subsequently filled in by the combined action of primase and polymerase (Fig. 1c). Greider and Blackburn (8, 9) have isolated and characterized a terminal transferase-like activity from *Tetrahymena* which can add T_2G_4 repeats to single-stranded primers which represent either the *Tetrahymena* or yeast GT-rich strands. The yeast enzyme must have the remarkable property of recognizing internal $C_{1-3}A$ repeats, even though it adds new DNA to the terminus of the DNA molecule.

The results obtained in this paper argue against recombinational models for telomere growth such as those proposed by Bernards et al. (2) and Walmsley et al. (34). Since nontelomeric linker sequences can be retained between the original C_4A_2 repeats and the newly added $C_{1-3}A$ repeats, there does not seem to be any opportunity for homologous strand invasion or strand slippage followed by extension. The non-template-directed DNA synthesis model is also applicable to the de novo addition of telomeres to the new ends generated during the processing of micronuclear chromosomes into macronuclear fragments during ciliate development.

The existence of the elongation reaction also provides a simple explanation for chromosome healing. This is the apparently spontaneous generation of telomeres at the termini of broken chromosomes (9, 17). We suggest that healing may occur when a CA-rich sequence in the correct orientation for the elongation reaction is exposed as a result of exonucleolytic degradation from a broken end. For instance, the generation of a telomere that lies at least 600 bp from the *Tetrahymena* end in A142p1 may reflect the presence of a CA-rich region (66% C's or A's in a 70-bp region) in the *HIS3* gene immediately adjacent to the *Bgl*II site which was used to create the substrate for the elongation reaction.

If the elongation reaction fulfills all the requirements for telomere replication, what then are the nature and role of the resolution reaction? In the Bateman (1) model for telomere replication, failure to resolve newly replicated telomeres fuses pairs of sister chromatids together as dicentric chromosomes. These dicentrics would lead to chromosome loss in two ways: either the dicentric is inherited intact by one daughter cell, leaving the sister cell without any copies of this chromosome, or the dicentric is broken in mitosis and some of the chromosome fragments are lost. If normal telomere replication involved the resolution reaction, its failure rate could not be greater than the frequency of chromosome loss, which has been measured at about 10^{-5} per cell per generation. We have measured the resolution frequency of an inverted repeat of *Tetrahymena* telomeric repeats at 10^{-2} per cell per generation. For the Bateman model to be correct, an inverted pair of yeast telomeric repeats would have to resolve at a frequency 10^7-fold greater than the *Tetrahymena* repeats. Furthermore, we have found that a pseudo-inverted repeat which is composed of a stretch of C_4A_2 repeats arranged head to head with a stretch of CA repeats is resolved much more slowly than the inverted repeat of C_4A_2 sequences. Since yeast telomeres consist mainly of CA repeats (26), the slow resolution of the pseudo-inverted repeat suggests that the yeast telomeric sequences are probably not better substrates for the telomere resolution reaction than *Tetrahymena* sequences. The slower resolution of the pseudo-inverted repeat does imply that the resolution reaction favors substrates which are symmetric about the center of the inverted repeat and therefore that the reaction proceeds by a cruciform intermediate. In particular, we suggest that any perfect inverted repeat in the yeast genome can form a cruciform structure at a low frequency. The base of the cruciform is identical to a Holliday junction and can therefore be cleaved by enzymes which recognize Holliday junctions, thus breaking the chromosome at the site of the inverted repeat (Fig. 1b). Only if the sequence forming the cruciform is a telomeric sequence will it be a substrate for telomere elongation and give rise to a new pair of telomeres.

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