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The fate of deleted DNA produced during programmed genomic deletion events in *Tetrahymena thermophila*

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ABSTRACT

Thousands of DNA deletion events occur during macronuclear development in the ciliate *Tetrahymena thermophila*. In two deleted genomic regions, designated M and R, the eliminated sequences form circles that can be detected by PCR. However, the circles are not normal products of the reaction pathway. The circular forms occur at very low levels in conjugating cells, but are stable. Sequencing analysis showed that many of the circles (as many as 50% of those examined) reflected a precise deletion in the M and R regions. The remaining circles were either smaller or larger and contained varying lengths of sequences derived from the chromosomal DNA surrounding the eliminated region. The chromosomal junctions left behind after deletion were more precise, although deletions in either the M or R regions can generate any of several alternative junctions (1). Some new chromosomal junctions were detected in the present study. The results suggest that the deleted segment is released as a linear DNA species that is degraded rapidly. This species is only rarely converted to the stable circles we detect. The deletion mechanism is different from those proposed for deletion events in hypotrichous ciliates (2–4), and does not reflect a conservative site-specific recombination process such as that promoted by the bacteriophage λ integrase (5).

INTRODUCTION

Ciliated protozoa provide examples of multiple types of programmed genomic rearrangements (6). These organisms have two nuclei, a micronucleus and a macronucleus. The micronucleus is transcriptionally inactive and is an analog of an inactive germ cell nucleus. The larger macronucleus is transcriptionally active and is an analog of a somatic nucleus.

Nutritional starvation induces cells of different mating types to undergo conjugation. The conjugants exchange haploid gametic nuclei to form diploid zygotic nuclei. Subsequent steps include mitotic divisions of zygotic nuclei, the products of which give rise to new micronuclei and macronuclei. Old macronuclei are gradually degraded. In *Tetrahymena thermophila*, the entire process is completed in about 20 h.

Macronuclear development in ciliates is accompanied by a variety of DNA rearrangements, including the elimination of a substantial portion of the micronuclear genome (7). In *T.thermophila*, about 15% of the genome is eliminated in the form of about 6800 site-specific deletion events, averaging 2 kbp in size and scattered about the genome. A striking feature of this type of DNA rearrangement is its high efficiency and precision. Deletion of all the MIC (micronucleus)-specific sequences occurs during a 2 h period of macronuclear development, commencing about 10–12 h after mating is initiated. The mechanism exploited to bring about genomic deletions is unknown.

Three site-specific deletions in *T.thermophila* have been characterized by Yao and co-workers (6). They occur within a short genomic region and are designated L, M, and R for left, middle, and right, respectively. The M region is the best-studied. Here, two different deletion events may occur, deleting either 600 or 900 bp (Figure 1B). The boundaries of the deleted segments are marked by 5 or 8 bp terminal direct repeats, although these have no identified role in the deletion process. Short polypurine tracts, found about 45 bp distal to each terminal repeat, are required for deletion and represent the only *cis*-acting sequences defined to date for a ciliate deletion reaction (8, 9). Their location defines the deletion endpoints in the M region (9). The polypurine tracts are absent in the neighboring R region, which also undergoes a deletion of 1.1 kbp (10).

A promising direction in the investigation of the mechanism of genomic rearrangements in ciliates is analysis of the products or byproducts that appear during the process. In *Euplotes crassus* the eliminated fragments derived from the deletion of IES elements or the transposon-like Tec1 and Tec2 elements can be detected as circles (11–13). The circle junctions contain two copies of the terminal direct repeat separated by a short segment of heteroduplex DNA derived from the chromosomal regions flanking the eliminated segment (2, 3). The data permitted the authors to propose a detailed model for the deletion mechanism. The deleted TBE1 elements of *Oxytricha trifallax* are also found as readily-detected circular species with defined structures (4).

We have begun an investigation of genomic deletions in the M and R regions during macronuclear development in *T.thermophila*. By means of PCR techniques we have detected circular forms of the eliminated sequences. However, the circles are found in much lower abundance than those observed in

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Euplotes or *Oxytricha*, and they are not intermediates on the usual reaction pathway.

MATERIALS AND METHODS

Cell lines and plasmids

Strains CU427.2VI and CU428.1VII were kindly provided by Peter Bruns. Cells were mated as described previously (14). Cell cultures used in these experiments had a mating efficiency of not less than 90%, as determined by microscopy.

Isolation of genomic DNA

The genomic DNA used in all experiments was isolated essentially as described (10), except that the lysate was adjusted to 2 M ammonium acetate and the DNA was precipitated with 3 volumes of ethanol. After a second ethanol precipitation, the final precipitate was redissolved in TE buffer (10 mM Tris−HCl, 1 mM EDTA, pH 8.0) at a concentration of 200 μg/ml.

Oligonucleotides

These were synthesized with an Applied Biosystems DNA/RNA Synthesizer A394. The oligonucleotides used as primers in PCR are:

ma1, 5′-TCTACGACAACCTATTGTACCACACAAT-3′;
ma2, 5′-AAAGCAAGAAGGCTACTTAGCTTTCAAATT-3′;
ma3, 5′-TATCAGTTCTCATCAAGTTGTAATGCTA-3′;
ma4, 5′-GAATTTCAATACCTGGATTTATCAATACAT-3′;
mb1, 5′-TTAATAAAGTAAAGAATGAACAAAATTAGC-3′;
mb2, 5′-TTGATGCCTTTTCTAATATTTTCAACTT-3′;
mb3, 5′-CTCTATCTATACAAACACAGTTGATGGT-3′;
mb4, 5′-TTTCCATCCTTGACTTAAAGAAAATCTCC-3′;
mc1, 5′-TGTTCCATATTAGAAAAATAAAAGTATCAGTA-3′;
mc2, 5′-GGTACGATAGATCGACTGACGGTTTTA-3′;
mc3, 5′-GGATAAATTTTAGAATAAACAACTCAATATGGC-3′;
mc4, 5′-GTAAATAATAAGGAACCTCTTACTGTGATA-3′;
md1, 5′-GTTTGTATTTCAATACTCTTAAAGACATAT-3′;
md2, 5′-GTTCAAATTTTCTAAATAAATTAAAGCAAA-3′;
ra1, 5′-CCTCAATTTACCTCATGTTGGCTATCT-3′;
ra2, 5′-CAAGACTAAATGTTTGATATATTTCTAACTC-3′;
ra3, 5′-CGGAAATACTTCGTTCATATTTATTTGTAT-3′;
ra4, 5′-TAATTCACGTAATCAAGGACTACTAATATT-3′;
rb1, 5′-GACGAAATAACAAAATGGTTTAAGCATACTTAC-3′.

PCR

PCR reactions were performed in 50 μl aliquots containing 1 μg genomic DNA, 0.4 μM each primer, dGTP, dATP, dTTP and dCTP at 0.2 mM each, 2.5 mM $MgCl_2$, 0.05 M KCl, 10 mM Tris−HCl (pH 9.0), 0.1% Triton X-100. The mixture was preincubated at 94°C for 3 min and at 55°C for 3 min to provide maximum specificity in the initial cycle of PCR ('hot start') (15). The mixture was centrifuged for several seconds to spin down condensed drops of evaporated water, and 0.2 μl (1 unit) *Taq* polymerase (Promega) was added. The mixture was covered with a layer of mineral oil and amplified by PCR for 35 cycles. Each cycle consisted of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Cycling was concluded with a final extension at 72°C for 5 min. In the 'nested primer sets' PCR modification, 3 μl of the PCR product was transferred into a new mixture, containing 2 μl of a 10 μM solution of each inner primer. Concentrations of dNTP, $MgCl_2$ and conditions of reaction were the same as above. The preliminary step ('hot start') and 35 cycles of PCR were carried out as above. A portion of PCR product (8 μl) was analyzed by electrophoresis in a 1.5% agarose gel containing 1 μg/ml of ethidium bromide.

DNA sequencing

PCR products were isolated from gel slices by incubation in 2 volumes of TE buffer under agitation at 37°C. The eluate was adjusted to 2 M ammonium acetate, and the DNA was precipitated with 3 volumes of cold ethanol. The precipitate was redissolved in TE buffer to a concentration of 100 μg/ml.

Direct sequencing of PCR products was carried out by the dideoxy nucleotide chain termination method with the following modifications (I.Goryshin, personal communication). Sequencing reactions contained 2 μl (200 ng) PCR product, 2 μl 10× KGB buffer (250 mM Tris−acetate, pH 7.6, 100 mM Mg acetate, 1 mg/ml bovine serum albumin, and 10 mM β-mercaptoethanol), 2 μl DMSO and 2 μl 10 μM oligonucleotide primer (the primers used were the same oligonucleotides used for amplification of a given PCR product). The final volume was adjusted to 14 μl with water. The mixture was boiled for 3 min, immediately quenched in liquid nitrogen, thawed at room temperature, and centrifuged for several seconds to spin down condensed liquid. To this was added 3 μl of a reaction mixture containing 2 μCi/μl [α-^{32}P]dATP (Amersham), Sequenase 2.0 (USB, 1 unit/μl), 0.6 μM each dGTP, dTTP and dCTP, and 20 mM DTT. After incubation at room temperature for 1 min, four 3.8 μl aliquots of the mixture were added separately to 2.5 μl of the ddG, ddA, ddT or ddC termination mixtures supplied by USB. The tubes were left at 37°C for 5 min. Then 4 μl of stop-solution (supplied by USB) was added to each. Sequencing products were denatured by boiling for 5 min and loaded on a sequencing gel.

Sequencing of M and R regions

To facilitate analysis of some of the DNA species detected in this study, the published sequence of a 1326 bp genomic segment containing the M region (16) was extended 827 bp to the left, and 787 bp to the right. The published R region sequence (10) was extended by 371 bp to the right. The plasmid pM2.1HX (4.3 kbp; a gift from M.-C.Yao), which contains a 2111 bp *Hin*dIII−*Xba*I fragment derived from *T.thermophila* encompassing the M region, was used to extend the sequence to the right. Genomic sequences corresponding to the left flank of the M region and the right flank of the R region were amplified by inverse PCR techniques (16). Sequencing was carried out as described above. We note that a genomic *Eco*RI site used in the inverse PCR procedure was found about 1 kbp away from the M1 repeat that marks the left end of the M region, ~3 kbp nearer to the M region than previously reported (17). We also detected two G→A base replacements located 67 and 76 bp to the left of M1. Three different PCR products derived from this region were sequenced, and all contained the base replacements. The apparent discrepancies probably reflect sequence polymorphisms in different laboratory strains of *T.thermophila*. The strain used to generate inverse PCR products for sequencing, CU428.1VII, is different from the strain used by Yao and co-workers (strain B1868-IV; ref. 4) to generate the original sequence. The sequence data are available from the GenBank data library under the accession nos U12971 (M region) and U12972 (R region).

RESULTS

Circular forms of eliminated sequences in conjugating cells of *T.thermophila*

Southern analysis failed to detect eliminated sequences after their deletion in conjugating cells of *T.thermophila* (18), suggesting that they are degraded rapidly. To improve sensitivity and

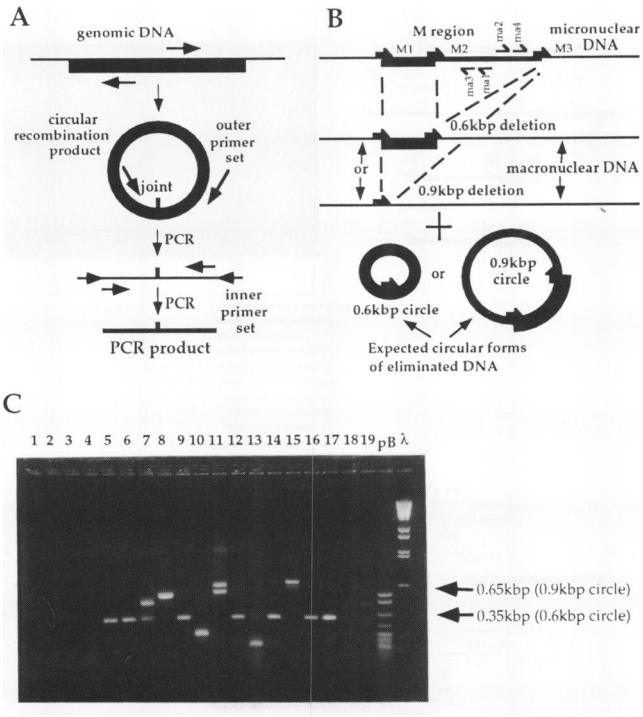


Figure 1. Circular forms of the sequences deleted from the M region. (**A**) The inverse PCR strategy used to detect circular DNA species derived from the M and R regions. Primers are targeted to sequences within the M or R regions, oriented so that they direct replication away from each other. A specific DNA segment is amplified only if the ends of the deleted region come together in a circle. Sensitivity is improved by incorporating two sequential PCR amplifications, using nested primers. (**B**) Deletion events in the M region. Deletions in the M region occur between terminal repeats labeled M2 and M3, or M1 and M3, resulting in 0.6 or 0.9 kbp deletions, respectively. PCR primers used in panel C are designated by arrows labeled ma1–ma4. (**C**) Detection of circular forms by inverse PCR. DNA was isolated from conjugating cells at 12 h after mixing (lanes 1–17) or from starved vegetative cells from strains CU427.2VI and CU428.1VII (lanes 18 and 19, respectively). The DNA analyzed in lanes 1–12 was from the same mating trial. Analyzed DNA in lanes 13–17 was from a second mating trial. Lanes 1–4 contain controls in which 2 of the 4 primers were used for PCR in 4 pairwise combinations: ma1 and ma2 (lane 1), ma3 and ma4 (lane 2), ma2 and ma3 (lane 3), ma1 and ma4 (lane 4). All 4 primers were used as a nested set in lanes 5–19. *pB* is a *Msp*I digest of pBR322 and λ is a *Bst*EI digest of bacteriophage λ DNA. Arrows at right indicate the expected sizes of PCR products derived from a 0.6 or 0.9 kbp excision and circularization of DNA from the M region, using the nested primers.

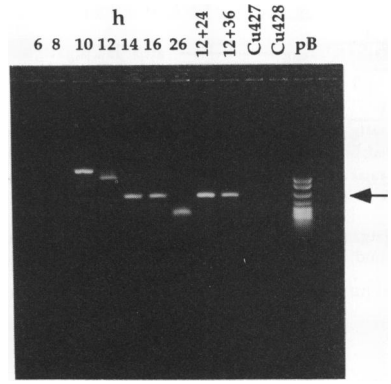


Figure 2. Temporal study of circle production in the M region. All the DNA preparations were analyzed with the same set of nested primers used in Figure 1. Time (h), beginning with initiation of conjugation, is designated above the gel lanes where appropriate. '12+24' and '12+36' DNA preparations were isolated from cells which were agitated at 200 r.p.m. beginning at 12 h after the initiation of conjugation, for 24 and 36 h, respectively. The two final lanes show a PCR analysis of DNA from starved CU427.2VI and CU428.1VII, agitated synchronously with conjugating cells for 36 h. *pB* is a *Msp*I digest of pBR322 DNA. The arrow indicates the 0.35 kbp PCR product expected with a circle derived from a 0.6 kbp deletion.

determine if deleted segments of the *Tetrahymena* genome are released as circles, we adopted the strategy of inverse PCR (Figure 1A) used by Tausta and Klobutcher (11) to detect circular forms of deleted segments in *E.crassus*.

The initial object of investigation was the M region, illustrated in Figure 1B. The two different deletions that occur in this region share the right boundary, designated M3. The search for circular deletion products was carried out 12 h after the initiation of conjugation, a time corresponding to the known DNA rearrangement period in the developing macronucleus of *T.thermophila* (18). However, we failed to amplify any PCR product in several attempts with a series of inverse primer pairs targeted to the 0.6 kbp deletion segment (Figure 1B, ma1–ma4; Figure 1C, lanes 1–4). Our failure could be attributed to an absence of circular forms, or to the presence of circles at concentrations too low to be detected by this method.

To improve sensitivity we used nested primer sets, a PCR modification described by Mullis and Faloona (19). Two primer sets, ma1/ma2 and ma3/ma4 were used sequentially to amplify the DNA sequences in two steps. With this method we detected PCR products in many separate trials, including multiple trials with a single *Tetrahymena* DNA preparation taken from cells 12 h after mating, and in additional trials on different DNA preparations from other matings (Figure 1C, lanes 5–17). As a control, DNA was prepared from starved vegetative cells (which are not undergoing genome rearrangements) and subjected to sequential PCR experiments with the nested primer sets. No amplified DNA species were detected (Figure 1C, lanes 18 and 19). The PCR product often had the expected size (~350 bp, Figure 1C), corresponding to a circular form derived from the 0.6 kbp deletion (Figure 1C, lanes 5, 6, 9, 12, 14, 16 and 17).

The circles were apparently present at very low levels. This conclusion is not based only on the extra sensitivity of the PCR method used. Figure 1C and subsequent figures present only those PCR trials that generated a product band. In experiments with the M region, only about half of the 1 μg aliquots from cells collected 12 h after conjugation was initiated produced a PCR product band. If the aliquot size was doubled, most (80–90%) of the aliquots produced a PCR product (data not shown). For this reason, the controls with DNA samples from starved cells (Figure 1C, lanes 18 and 19) were repeated more than 20 times, with identical results, to reach the conclusion that the circles were specific to conjugating cells.

Unexpectedly, a number of the PCR products (about 50% of the total in this experiment, higher percentages in some experiments described below) were heterogeneous in size (Figure 1C, lanes 7, 8, 10, 11, 13 and 15). It was striking that different aliquots of the same DNA preparation gave rise to different PCR products (Figure 1C, lanes 7, 8, 10 and 11). In a few cases, two different PCR products were amplified from a single aliquot (lanes 7 and 11). This suggested that some of the circular forms of the deleted sequences in conjugating *Tetrahymena* cells were variable in size, and again indicates that the concentration of the

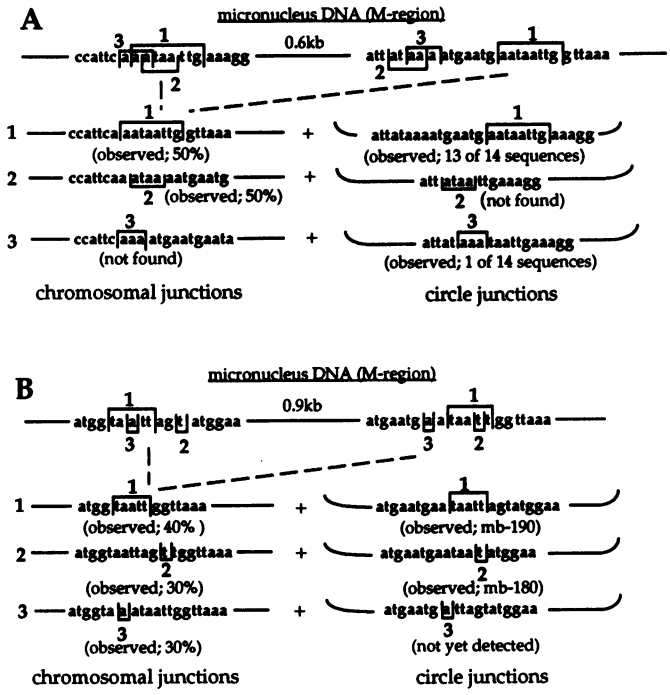


Figure 3. Chromosomal and circle junctions created by deletion in region M. Direct terminal repeats bordering each observed deletion (reflecting the observed structure of circle junctions, chromosomal junctions, or both) are bracketed. The chromosomal and circle junctions were analyzed in DNA from conjugating cells in a single mating at 16 h after initiation of conjugation. All the junctions were analyzed by direct sequencing of PCR products. The paired chromosomal and circle junctions (sets 1−3) are those that would be derived from a conservative and reciprocal recombination process. (**A**, **B**) Junctions detected for the 0.6 and 0.9 kbp deletion reactions, respectively.

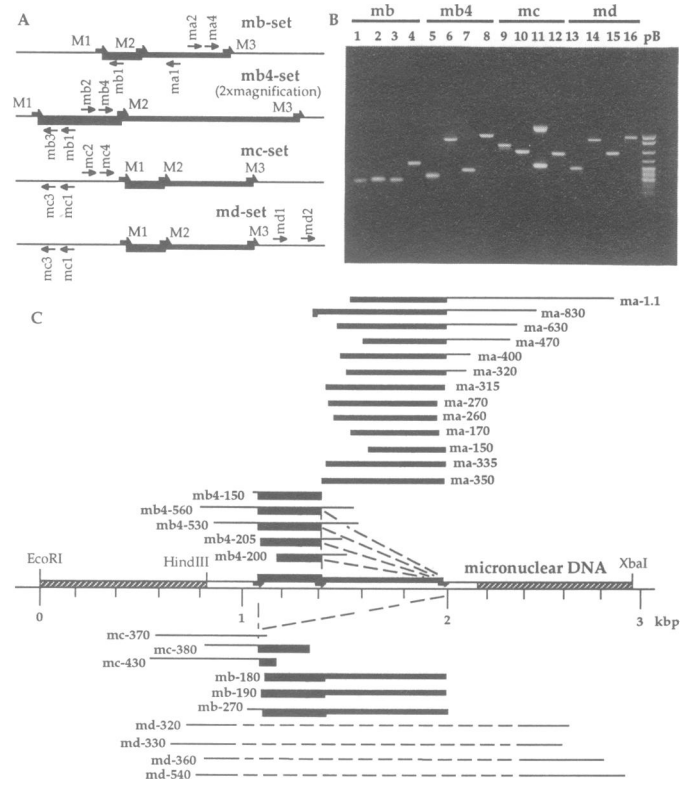


Figure 4. Further analysis of circles derived from the M region. (**A**) Primer sets used in the study. (**B**) Analysis of circle variability. DNA isolated from conjugating cells (a single mating) at 12 h after mixing was used in all PCR trials. The primer sets used in each trial are designated above the lanes. *pB* is a *Msp*I digest of pBR322 DNA. (**C**) Maps of analyzed recombination products. In the center, a map of the micronuclear M region locus is presented. Hatched boxes are regions sequenced during the course of this study. Solid black regions are the deleted segments. Circular recombination products are shown in linear form in relation to the M region. The designation of each detected recombination byproduct reflects the primer set used for detection and the length of the corresponding PCR product (for example, the ma-400 circle was generated with the ma primer set, and the length of the PCR product is 400 bp). The dashed line in the map of md circles indicates sequences that may be part of the circles, but not part of the PCR product because of the primer set design.

circles is so low that each aliquot contains no more than one or a few circles. Each aliquot contains DNA derived from about 10^6 cells. Circle variability was revealed in all the conjugating cultures we studied. In the continued discussion below, we refer to circles that did or did not give rise to PCR products of the expected sizes as precise or odd-sized, respectively. In this context, 'precise' is defined only within the limits of resolution of gels such as that in Figure 1C.

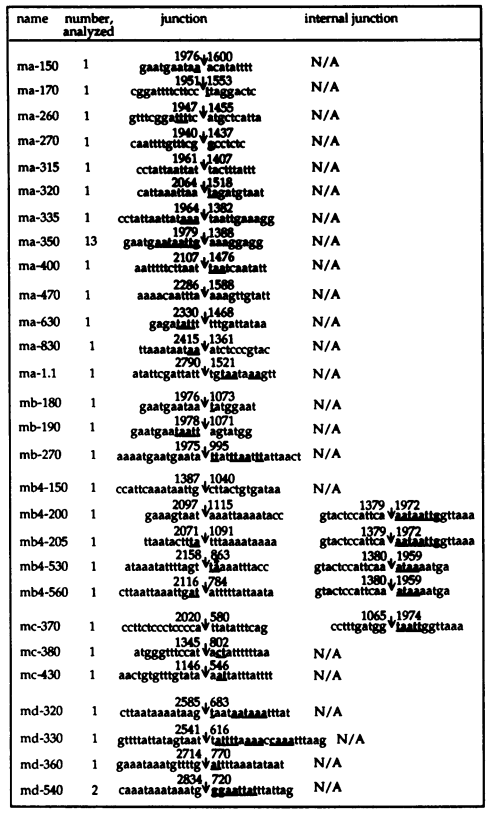
Temporal study of the production of circular forms of deleted sequences

The circles appear no sooner than 10 h after the beginning of conjugation (Figure 2). This time coincides well with the time of the beginning of the DNA rearrangement period (18), again associating the circular forms with the programmed recombination events in conjugating cells of *T.thermophila*.

The very low concentration of the circular forms of eliminated sequences in conjugating cells of *T.thermophila* might be explained by a very high rate of degradation. However, the circles proved to be quite persistent. The genomic rearrangement period was shown to be completed 14−16 h after the beginning of conjugation (18), whereas the circles were found at 26 (Figure 2) and even 100 h (data not shown) after mixing the cells. The cells were not refed at any time in this experiment.

Circles were detected more frequently at the later timepoints (e.g. PCR products were found in virtually all trials using DNA from cells collected at 16 h and later times after the initiation of conjugation), suggesting an accumulation of circles with time. The proportion of the detected circles which reflected a precise deletion also increased at later timepoints, and the odd-sized circles were observed only occasionally. One survey found that 9 of 20 circles detected between 10 and 14 h reflected a precise deletion, while 24 of 26 circles found between 16 and 26 h were precise. As noted in the Discussion, an accumulation of circles should lead to a selective amplification of the 'precise' deletion circles, which would predominate in any mixture. The use of primers targeted to regions outside the 0.6 kbp deletion permitted the ready detection of odd-sized circles in samples from later timepoints in which the use of ma1−ma2 + ma3−ma4 primers produced precise circles almost exclusively. This suggests that the odd-sized circles are present in all of the samples, but are readily detected at later timepoints only when the sample is dilute or special methods are used which permit their amplification.

To check the possibility that the circles were unstable, but produced at late times by the few cells which entered into conjugation late, a cell culture was subjected to strong agitation,



name	number, analyzed	junction	internal junction
ma-150	1	1976↓1600 gaatgaataa↓acatatttt	N/A
ma-170	1	1951↓1553 cggattttcttcc↓taggactc	N/A
ma-260	1	1947↓1455 gtttcggatttttc↓atgctcatta	N/A
ma-270	1	1940↓1437 caattttgttttcg↓gcctctc	N/A
ma-315	1	1961↓1407 cctattaattat↓tacttattt	N/A
ma-320	1	2064↓1518 cattaaattaa↓tagatgtaat	N/A
ma-335	1	1964↓1362 cctattaattataaa↓taatgaaagg	N/A
ma-350	13	1979↓1388 gaatgaataattg↓aaaggagg	N/A
ma-400	1	2107↓1476 aatttttcttaat↓taatcaatatt	N/A
ma-470	1	2286↓1588 aaaacaatttta↓aaagttgtatt	N/A
ma-630	1	2330↓1468 gagataattt↓tttgattataa	N/A
ma-830	1	2415↓1361 ttaaataataa↓atcicccgtac	N/A
ma-1.1	1	2790↓1521 atattcgattatt↓tgtaataaagtt	N/A
mb-180	1	1976↓1073 gaatgaataa↓tatggaat	N/A
mb-190	1	1978↓1071 gaatgaataatt↓agtatgg	N/A
mb-270	1	1975↓995 aaaatgaatgaata↓ttatttaattattaact	N/A
mb4-150	1	1387↓1040 ccattcaaataattg↓cttactgtgataa	N/A
mb4-200	1	2097↓1115 gaaagtaat↓aaattaaaatacc	1379↓1972 gtactccattca↓aataattggttaaa
mb4-205	1	2071↓1091 ttaatacttta↓tttaaaataaaa	1379↓1972 gtactccattca↓aataattggttaaa
mb4-530	1	2158↓863 ataaatattttagt↓taaaatttacc	1380↓1959 gtactccattcaa↓ataaaatga
mb4-560	1	2116↓784 cttaattaaattgat↓attttattaata	1380↓1959 gtactccattcaa↓ataaaatga
mc-370	1	2020↓580 ccttctcccctcccca↓ttatatttcag	1065↓1974 cctttgatgg↓taattggttaaa
mc-380	1	1345↓802 atgggttttccat↓actatttttttaa	N/A
mc-430	1	1146↓546 aactgtgtttgtata↓aattatttatttt	N/A
md-320	1	2585↓683 cttaataaaaataag↓taataataaatttat	N/A
md-330	1	2541↓616 gttttattatagtaat↓tatttttaaaaccaaatttaag	N/A
md-360	1	2714↓770 gaaataaatgttttg↓attttaaatataat	N/A
md-540	2	2834↓720 caaataaataaatg↓ggaattatttattag	N/A

Figure 5. Sequence analysis of M-circle junctions. Numbers above each junction indicate the distance of bases linked at the junction from the *Eco*RI site shown in Figure 4C. An internal junction is created by internal 0.6 or 0.9 kbp deletion in the large circles.

beginning at 12 h after initiation of conjugation, to quantitatively disrupt further mating (14). The PCR products from the M region were found at similar frequencies after 24 and 36 h of agitation (Figure 3), though we did not observe mating pairs of cells in the agitated cultures (data not shown). As a control to determine if agitation results in anomalous circle generation, we agitated starved cells just as we had done with the conjugating cells. However, we did not find any circles in the starved cells in more than 10 separate trials (Figure 2). We concluded that the circular forms of eliminated sequences in conjugating cells of *T.thermophila* are stable but inefficiently produced byproducts of the deletion reaction.

Sequence analysis of circular species eliminated from the M region

To further characterize the PCR products, and to determine if they were all derived from the M region, 41 of the amplified PCR products, chosen more or less at random to represent all observed classes, were subjected to sequence analysis. First we directly sequenced 14 of the PCR products corresponding in size to a circle expected from a precise 0.6 kbp deletion. Thirteen of these contained one copy of the 8 bp direct repeat bordering the corresponding eliminated segment (Figure 3, circle 1). The fourteenth circle had a junction corresponding to a deletion of sequences flanked by a repeat of three A residues; these deletion endpoints are only slightly removed from the normal ones (Figure 3, circle 3). There were no additional sequences derived from DNA flanking the deleted sequences in these circle junctions.

We also examined the chromosomal junctions left behind after a 0.6 kbp deletion. Unlike the circles, the chromosomal junctions were readily detected by standard PCR protocols. PCR amplification of a short region spanning the expected junction generated two products, which differed in size by 13 bp (data not shown). Both were isolated and sequenced. One proved to be the junction previously described by Austerberry and Yao (1) (Figure 3A, chromosomal junction 1). The other was a junction not previously detected (Figure 3, chromosomal junction 2).

Although the new chromosomal junction was readily detected in each experiment, none of the 14 analyzed 0.6 kbp circle junctions corresponded to it. This was one of several observations indicating that there was no simple reciprocal relationship between circles and chromosomal junctions.

We next focused on the 0.9 kbp deletion in the M region. Outer primers ma1 and ma2 in combination with inner primers mb1 and ma4 (Figure 4A) were used to selectively amplify the junction site of circles produced by deleting the 0.9 kbp segment. Two circles whose size appeared to correspond to a precise 0.9 kbp deletion were analyzed (Figure 3B, circles 1 and 2). Only the first of these corresponded to a previously reported chromosomal junction (1).

PCR amplification of chromosomal junctions formed as a result of the 0.9 kbp deletion generated a single band, but sequencing revealed a complex mixture of at least 3 different junctions. To isolate individual junctions, the PCR products were serially diluted by a factor of 10^{14}. After reamplification and sequencing, the three chromosomal junctions in Figure 3B were found. One of these (junction 2) had not been previously reported.

We next analyzed the PCR products that were significantly larger or smaller than expected, using additional sets of primers. All primer sets generated PCR products of variable size (Figure 4B). Many of these were sequenced, and all were found to be derived from the M region. The additional DNA in the larger circles was always derived from chromosomal DNA immediately flanking and contiguous with the deleted region. The amount of flanking DNA included in the circle was variable, often involving hundreds of base pairs. The circle endpoints (defined by the circle junction) usually, but not always, occurred within short repeated sequences (from 1 to 15 bp in length) (Figure 5). The relationship of these larger circles to programmed chromosomal deletion events is underscored by the fact that a number of them have one of the normal boundaries and contain flanking DNA from only one side of the deleted region (e.g. circle mb-270, Figures 4C and 5).

Some circles had flanking DNA derived from both sides of the M region (e.g. circles mb4-560, mb4-530, and mc-370, Figure 4C). Several of the larger circles had an internal deletion precisely corresponding to the chromosomal 0.6 or 0.9 kbp deletions (mb4-200, mb4-205, mb4-530, mb4-560, mc-370 in Figure 4C). This indicated that the circular byproducts we were analyzing could themselves become substrates for the deletion reaction. Internal deletions occurred in *all* of the circles that included the *cis*-acting polypurine tracts at both ends. If the circle had no polypurine tract on one side, the internal deletion was not observed (for example, ma-830 in Figures 4 and 5). The mc-370 circle seems to be an exception to this general rule. This circle has an internal 0.9 kbp deletion, though one of the normal polypurine tracts (at right) is absent. In this one case, circularization of the recombination product introduced a new

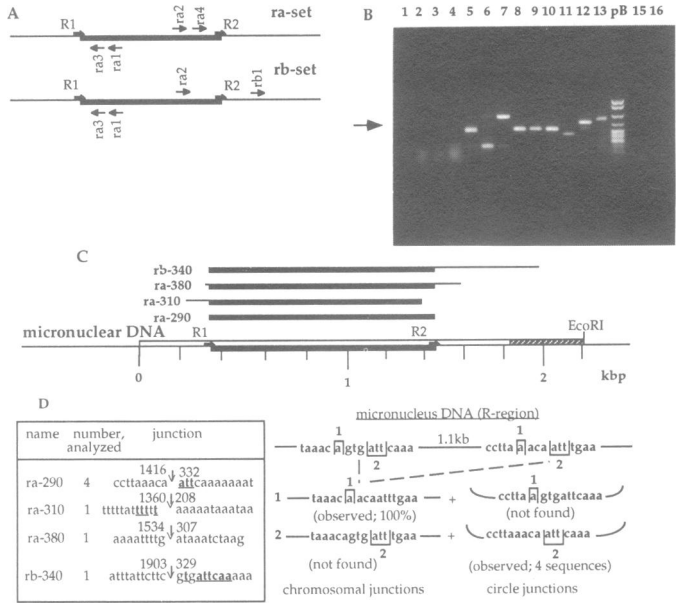


Figure 6. Circles derived from deletion in the R region. (**A**) Primer sets used in the study. (**B**) PCR analysis of circles generated from the R region in conjugating cells. The DNA analyzed was isolated from conjugating cells in a single mating at 12 h after initiation of conjugation (lanes 1–13). The first 4 lanes are controls showing typical results obtained when any two of the ra primers are used in pairwise combination; ra1 and ra2 (lane 1), ra3 and ra4 (lane 2), ra2 and ra3 (lane 3), ra1 and ra4 (lane 4). The ra primers were used in the nested modification (lanes 5–12) to detect circles. Lane 13 shows a PCR trial with the rb primer set. Starved cells of CU427.2VI and CU428.1VII were analyzed with the ra set (lanes 15 and 16, respectively). *pB* is a *Msp*I digest of pBR322. The arrow indicates the 0.29 kbp PCR product derived from the 1.1 kbp circle that represents a precise deletion. (**C**) Map of four circles derived from the R region based on sequence analysis. (**D**) Sequencing analysis of circle junctions. Numbers above the junction arrows reflect the distance in bp from the left flank of the sequenced region as presented in (C).

polypurine tract (5′-AAATATAA-TGGGGAGGGA-3′) within the circle junction. We hypothesize that some part of this tract effectively replaced the destroyed one, and, together with the left tract, permitted a 0.9 kbp deletion inside the circle.

An internal deletion in these circular byproducts creates a new junction in the circle that is analogous to a chromosomal junction. Notably, the 0.6 kbp deletion in 2 of the 4 analyzed large circles (Figure 5, mb4-530 and mb4-560) created the new type of chromosomal junction (chromosomal junction 2, Figure 3A) described above. This provides additional evidence that the new chromosomal junction occurs at a significant frequency as a product of 0.6 kbp deletions.

Although some microheterogeneity is evident in the chromosomal junctions as described above, we found no evidence for the kind of size and junction variability that was evident in the circle junctions.

Circular byproducts generated from the R region

Detection of circular forms of sequences eliminated from the R region again required the use of a nested primer set (Figure 6A and B, lanes 5–13). No circles derived from the R region were detected in DNA derived from starved cells in 7 separate trials. Many of the PCR products corresponded to the size expected (a PCR product of 290 bp) for the normal 1.1 kbp chromosomal deletion in the R region (10) (Figure 6B, lanes 5 and 8–10). Other circles were of variable size (Figure 6B, lanes 6, 7 and 11–13). We sequenced a number of the PCR products. The 1.1 kbp circles (Figure 6C and D, circle ra-290) corresponded to a precise deletion between the two 5′-ATT-3′ repeats, bordering the previously described deletion in the R region (1). Junctions of all four sequenced 1.1 kbp circles contained one copy of this repeat (Figure 6D, ra-290). The only chromosomal junction we detected was one previously reported (1), derived from an apparent exchange 3 bp away from the ATT repeat (Figure 6D). This is another case in which the major circle and chromosomal junctions cannot be derived from a reciprocal recombination event. As in the M region, the extra DNA in the odd-sized circles was always derived from flanking chromosomal DNA sequences.

Controls for potential PCR artifacts

We were alert to the possibility that the odd-sized circles could be PCR artifacts. The sequences of the circles eliminates the possibility that they are non-specific PCR products generated by amplification of some other region of the genome.

Another potential problem in PCR is template switching (15). For example, a partially extended rightward primer could, in a subsequent PCR cycle, anneal to a complementary sequence to the left of a leftward primer and be extended to create an artifactual circle junction. However, no circles derived from the M or R regions, odd-sized or otherwise, were found in starved cells or conjugating cells prior to the 8 h timepoint in over 50 separate trials with a variety of nested sets of inverse primers. The junctions identified in some of the odd-sized circles occurred at positions in which there was no homology to support the annealing step of template switching. Finally, almost all of the odd-sized circles are different, indicating that there are no special sequences in the M or R regions that facilitate template switching.

DISCUSSION

The primary conclusions of this study are: (a) the DNA deleted in the M and R regions during macronuclear development in *T.thermophila* can be detected in circular form, but the circles are not the usual products of the reaction pathway; and (b) there is somewhat greater microheterogeneity in the macronuclear junctions left behind by deletion than previously reported. Both properties suggest that there are at least some mechanistic distinctions between the M and R region reactions and the deletions characterized to date in hypotrichous ciliates (2–4). The results are also inconsistent with a conservative site-specific recombination process such as that mediated by the bacteriophage λ integrase or FLP recombinase (5).

The sequences of the circles indicate that they are derived from the M and R regions, and their appearance exhibits a clear temporal relationship to macronuclear development. However, the circles are present at very low levels and detection requires an extremely sensitive PCR technique. The circles are demonstrably stable, arguing against the possibility that they are formed in the usual deletion pathway but degraded rapidly. Together, these results suggest that the deleted DNA segments are initially released in a linear form that is degraded rapidly. In principle, release in other forms (such as a nicked circle) is not ruled out by the data, as long as degradation of the released DNA is rapid enough to preclude its detection. In very rare instances the deleted DNA is ligated or repaired to form the stable

circles we have detected. The similarities in the abundance and properties of the circles derived from the M and R regions suggest a common overall mechanism for DNA deletions, even though the regions share no obvious sequence identities.

In the M region, we identified one new chromosomal junction each for the 0.6 and 0.9 kbp deletion reactions. The observed endpoints of the alternative deletions fell within very short segments of the micronuclear DNA, spanning no more than 12 bp. There were no obvious sequence identities shared among the different sets of endpoints, reinforcing the conclusion of Godiska *et al.* (9) that sequences somewhat removed from the deletion ends play the major role in defining the boundaries.

We also sequenced a large number of circle junctions that had a size consistent with a precise deletion. Some of these corresponded closely to the chromosomal junctions, in the sense that they could have been derived from a reciprocal recombination process. However, many of the circle junctions had no counterpart among the chromosomal junctions, and vice versa.

As this work was being completed, we became aware that related observations had been made by Yao and Yao (20). Although the larger circles were not detected in their study, their data and conclusions drawn from it are in broad agreement with our own. In particular, they concluded that the deleted segments are initially released as linear DNA molecules and that the circles are reaction byproducts.

Although there is extensive overlap, both studies found a few precise circle or chromosomal junctions not found in the other study. This suggests that additional junctions may be present that escaped detection, or that some variability in the preferred routes of deletion exists from one laboratory strain to another. A significant degree of microheterogeneity in macronuclear junctions is evident in all of the deletion events characterized.

The major difference between the two studies lies in our detection of circles that include DNA flanking the deleted region. As detailed in Results, the frequency with which these circles were detected in our study and the sequences of the circles provide evidence that this observation is not an anomaly. We also carried out an extensive set of controls to rule out known artifacts that can arise in PCR. We believe that the apparent discrepancy is best explained by differences in methodologies used to generate DNA for analysis. Yao and Yao purified macronuclear anlagen DNA, which should be enriched for the circles relative to the whole cell DNA we used. Since almost all of the odd-sized circles were unique, they would not be readily detected in a mixture in which half or more of the circles were derived from precise deletions. We believe the odd-sized circles were detected only because many of our samples contained no more than one circle. There was an apparent decline in the frequency with which odd-sized circles were observed at later times after the initiation of conjugation, which we attribute to the accumulation of stable circles in the population of conjugating cells. We don't know how the odd-sized circles are generated, or whether their existence has mechanistic significance for the deletion process.

The very low abundance of circles indicates that the deleted DNA segment is probably released as a linear DNA segment in the usual reaction pathway. The structure of the chromosomal junctions is an important parameter that will help define the reaction that generated them. The circles we have characterized, odd-sized or otherwise, are rare reaction byproducts in this system. Further study is required to evaluate any information they may reveal about the deletion mechanism.

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