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Detection of circular excised DNA deletion elements in *Tetrahymena thermophila* during development

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ABSTRACT

Extensive programmed DNA deletion occurs in ciliates during development. In this study we examine the excised forms of two previously characterized deletion elements, the R- and M-element, in *Tetrahymena*. Using divergently oriented primers in polymerase chain reactions we have detected the junctions formed by joining the two ends of these elements, providing evidence for the presence of circular excised forms. These circlar forms were detected in developing macronuclear DNA from 12 – 24 h after mating began, but not in micronuclear or whole cell DNA of vegetative cells. They are present at very low abundance, detectable after PCR only through hybridization with specific probes. Sequence analysis shows that the circle junctions occur at or very near the known ends of the elements. There is sequence micro-heterogeneity in these junctions, which does not support a simple reciprocal exchange model for DNA deletion. A model involving staggered cuts and variable mismatch repair is proposed to explain these results. This model also explains the sequence micro-heterogeneity previously detected among the junction sequences retained in the macronuclear chromosome.

INTRODUCTION

Programmed DNA deletion occurs in many ciliated protozoa to eliminate specific DNA segments during macronuclear differentiation. These processes follow specific developmental programs and occur at thousands to tens of thousands of specific sites in the genome (1). Their wide occurrence and well-regulated nature suggest an important biological role and an intriguing mechanism, about which very little is known.

In this study we analyze the deletion process in the holotrichous ciliate *Tetrahymena thermophila*, with the hope of better understanding its molecular mechanism. Roughly 6,000 DNA segments are deleted in *Tetrahymena* (2). They range in size from several hundred to several thousand base pairs (bp), contain both unique and repetitive sequences (3), and are distributed widely in the genome (4, 5). The complete DNA sequences of three such deletion elements (6–8) and partial sequences of two others (9, 10) have been reported. They share very little sequence identity and lack features typically associated with mobile genetic elements, such as long terminal direct or inverted repeats, or apparent protein coding regions. The most prominent common feature is the presence of terminal direct repeats of 1–8 bp in length, but they are apparently not essential for the deletion process (11).

We have previously carried out analysis of two neighboring deletion elements, the M- and R-elements, in *Tetrahymena* (previously referred to as the M and R regions of clone cTt455) (2) (Figure 1). The M-element is deleted in two alternative forms in roughly equal frequency: a 0.9 and a 0.6 kb form which share the same right boundary (3). An 8 bp direct repeat marks the left and right boundaries (M2 and M3 respectively, Figure 1) of the 0.6 kb form, 5 bp of which are also located at the left boundary of the 0.9 kb form (M1, Figure 1) (7). Deletion of the 0.6 kb form is precise, resulting in the removal of one copy of the 8 bp repeat. Deletion of the 0.9 kb form is somewhat variable, resulting in the removal of the 5 bp repeat only in roughly half of the cases examined (12). A transformation system for *Tetrahymena* has been developed in this laboratory to analyze the *cis*-acting sequences of this process. These studies show that a polypurine sequence (5′-AAAAAGGGGG or close variant thereof) located in the flanking regions ~45 bp away from all three deletion boundaries plays a critical role (13). It acts alone to determine the posititons of the deletion boundaries in a distance- and orientation-specific manner (11).

The R-element is 1.1 kb in size and contains only a single repeated nucleotide (A) at each boundary, although a pair of 6 bp repeats is located nearby. Deletion of this element produces a junction sequence containing one of the terminal A nucleotides in most cases studied (6). There is no sequence resembling the 5′-AAAAAGGGGG motif near the element boundaries. Some other flanking sequences apparently specify the deletion boundaries in a similar distance-dependenet manner (D.Chalker, A.La Terza, A.Wilson and M.-C.Yao, unpublished observations).

Despite this knowledge, much remains unknown about the mechanism of DNA deletion in *Tetrahymena*. For instance, it is not known whether the DNA is deleted as an intact piece, and if so, whether it is removed as a linear or circular form. It is

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also not known what type of cleavage occurs at the element's ends. Studies in two other ciliates have provided information in this regard. In the hypotrichous ciliate *Euplotes crassus* roughly 60,000 different pieces of DNA (referred to as IES, for internal eliminated sequence) are deleted in a manner similar to that in *Tetrahymena* (14). Studies of two such elements have revealed circular excised forms during nuclear differentiation. These circles contain the elements with their two ends joined together, and an extra 10–16 bp sequence at the junction derived from the flanking region. Circular excised forms with an extra 10 bp sequence at the junction have also been observed for a transposon-like deletion sequence Tec1 in *Euplotes* (15, 16). These structures suggest a mechanism involving staggered cuts at the deletion boundaries (16, 17). In another hypotrichous ciliate, *Oxytricha trifallax*, a transposon-like element, TBE1, is also deleted during development (18). A circular excised form of this element has been detected, but it does not contain extra flanking sequences (19).

It has been shown previously that the sequences deleted in *Tetrahymena* during development are rapidly degraded. No putative excised form has been detected by genomic hybridization (3, 20). However, using the more sensitive polymerase chain reaction (PCR) method, we are now able to detect circle junctions corresponding to the excised M- and R-elements. In contrast to the results obtained from *Euplotes*, these junctions do not contain extra nucleotides. Instead, there is sequence micro-heterogeneity at two of the junctions reported here. A model involving staggered cuts and mismatch repair is proposed to explain these results. This model also accounts for the sequence micro-heterogeneity previously observed among the deletion junctions found in the macronuclear chromosome.

MATERIALS AND METHODS

Cells and cultures

Tetrahymena thermophila inbreeding lines CU427, CU428, B1868II, B1868VII and B* were obtained from Peter Bruns of Cornell University. They were cultivated in peptone media as described before (21). Strains CU427 and CU428 were used for large scale synchronous mating as described before (22). Cells were grown to late log phase ($2-3\times10^5$/ml), washed and starved in 10 mM Tris–HCl (pH 7.4), and mixed to start conjugation. Mating cells were checked under a microscope to determine pairing efficiencies, which ranged from 70–90%.

Nuclei and DNA isolations

Developing macronuclear anlagen were isolated from conjugating cells at 12, 14, 16, 18 and 24 h after cell mixing by sedimentation in a sucrose gradient under gravity (23). Contamination from macronuclei and micronuclei were determined by examination under a microscope, and was estimated to be 20% or less in DNA content. Micronuclei were isolated from vegetative cells grown to late log phase ($2-3\times10^5$/ml) by differential centrifugation and filtration (21). Contamination from macronuclei was ~15% in DNA content. DNA was isolated from purified nuclei by lysis in sarkosyl and equilibrium sedimentation in a CsCl gradient as described before (24). Whole cell DNA was purified from late log or stationary phase vegetative cells by SDS lysis, protease digestion and phenol extraction following a previously described method (6).

DNA cloning and characterization

Agarose gel electrophoresis, DNA transfer and hybridization were carried out using standard methods (25). Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer and were labeled at 5′ ends with ^{32}P for use as hybridization probes. PCR products were ligated to *Sma*I-digested pUC19 vector DNA and used to transform *E.coli*. Desired clones were identified by colony hybridization. For cloning of the M-element PCR products, secondary PCR amplification was performed using the same set of primers to increase the amount of material for cloning. Sequences of the cloned DNA were determined by using a modified T7 polymerase (Sequenase, United States Biochemicals, Inc.) in a chain termination method.

Polymerase chain reactions and oligonucleotides

PCR was carried out using Taq DNA polymerase and reaction buffer purchased from Perkin Elmer Cetus and the reaction conditions specified by the company. Each 30 μl reaction contained about 0.1–0.2 μg of anlagen or micronuclear DNA, or roughly 1μg of whole cell DNA as a template, and 0.2 μg of each of the primers. The enzyme was kept in a separate layer from the rest of the reaction components by paraffin until the tube was heated to start the reaction. The reactions were carried out at 94°C for 1 min, 53°C for 2 min and 72°C for 1.5 min for 40 cycles in a Perkin Elmer Cetus DNA thermal cycler. 5 μl of each reaction was analyzed by electrophoresis in a 1.5% agarose gel. The gel was blotted to a nitrocellulose filter under pressure and used for hybridization. Sequences (starting from 5′ end) of the oligonucleotides used as primers and probes are as follows: RI5-519RC: TTTAATTAGTCAGGTTATAGG; RI3-1317: CCTTAATTCACGTAATCAAGGAC; RI5-482RC: TTAACGGAAATACTTCGTTC; JRE1: CTTCCTTAAACAATTCAAAA; JRE2: CTTCCTTAAGTGATTCAAAA; M619R: TTAGAGAGGCTGGTCTCC; M1001: AACTTATTGAAATTCGGC; M601R: TTATTCAATTTTAAATAAAGTACA; K861M: TGGTACCTAAAGACCCAAGTTATTAG; M1022: CATTATGTATCCAGTCAGCAG; M299RC: CTATACAAACACAGTTGATGG.

RESULTS

Detection of the R-element junctions

In previous experiments using a genomic hybridization method we were unable to detect any excised form of the M- or R-deletion elements in developing macronuclei of *Tetrahymena*. These results suggested that these elements were degraded during or soon after deletion (3). Since this method is not very sensitive, it remains possible that some excised elements are present and may be detectable by the more sensitive method of polymerase chain reaction (PCR). We investigated this possibility by looking for circularized forms of the R-element. Two oligonucleotides (RI5-519RC and RI3-1317, primers 1 and 2 respectively in Figure 1) containing sequences within the R-element and oriented away from each other were used as primers in PCR. Macronuclear anlagen DNA isolated from synchronous mating cells at different stages of development, as well as whole cell or micronuclear DNAs from vegetative cells, were used as templates. A 374 bp fragment is expected if circular excised forms exist. In all trials the PCR products migrated as multiple bands with no clear and consistent pattern after agarose gel electrophoresis (data not

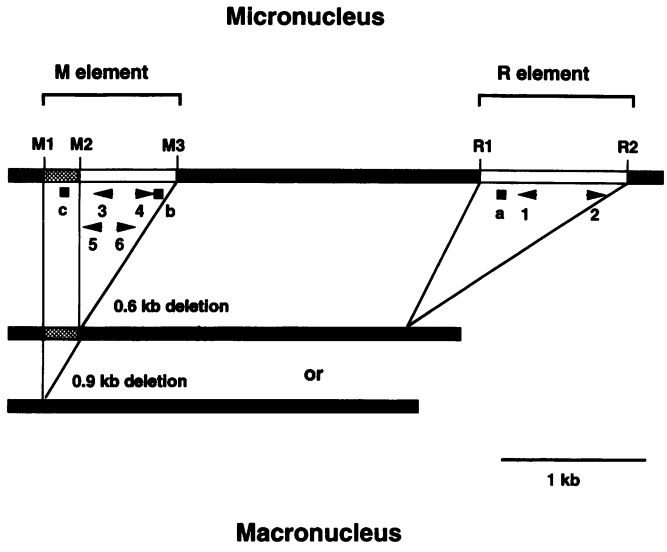

Figure 1. Deletion of the M- and R-element in *Tetrahymena*. This diagram illustrates the structural changes in DNA resulting from deletion of the neighboring M- and R-elements in *Tetrahymena*. The upper bar represents micronuclear DNA and the bottom two bars represent the two alternative forms of the macronuclear DNA generated during development. The open sections are deletion elements. The shaded section is deleted only in the 0.9 kb form of the M-element. M1, M2, M3, R1 and R2 mark the boundaries of these deletion elements. Arrowheads and squares under the bars indicate the approximate locations of the oligonucleotide sequences used as PCR primers (arrowheads) and hybridization probes (squares) in this study. Directions of the arrowheads indicate the 5′→3′ direction of the oligonucleotides.

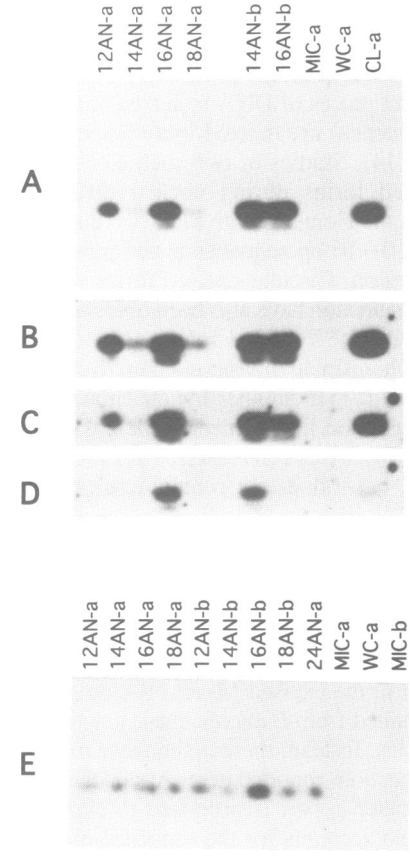

Figure 2. Detection of the excised R-element by PCR. Various *Tetrahymena* DNAs were used as templates in PCR to detect the circular excised form of the R-element. Five μl of each reaction was analyzed by electrophoresis in a 1.5% agarose gel. The gel was blotted and hybridized with an oligonucleotide probe to detect the specific products. Each lane (except CL-a) contains products generated using a different template DNA, and is specified above each lane. AN indicates anlagen DNA, and the preceding number indicates the time in hours after mating began that the anlagen were isolated. The lower case letters -a and -b indicate two different samples. Thus 12AN-a and 12AN-b are two anlagen DNA samples isolated from different populations of cells 12 h after mating had begun. MIC-a and MIC-b indicate micronuclear DNAs isolated from strain CU427 and CU428 respectively during vegetative growth. WC-a indicates whole cell DNA isolated from strain CU427 during vegetative growth. CL-a is a cloned plasmid DNA which contains the excised R-element sequence produced in a previous PCR reaction. It serves as a positive control for hybridization as well as a size marker. The clone was digested with *Hin*dIII and *Eco*RI to separate the insert from the vector. Approximately 0.1 μg of this DNA was loaded in the gel. (A–D) Different hybridization results of the same gel blot. Results in (**A**) are from hybridization with the oligonucleotide RI5-482RC (probe a in Figure 1). After exposure this filter was stripped of the probe and re-hybridized with the oligonucleotide JRE1 to generate the results in (**B**). JRE1 contains the junction sequence of the excised R-element circle determined in this study. This junction sequence is also present in the clone CL-a. These hybrid molecules remain stable under high stringency wash conditions (0.5×SSPE, 37°C). (**C, D**) The results obtained using hybridization probe JRE2 after JRE1 hybrids were removed from the filter. JRE2 contains a predicted element junction sequence (see Figure 3). The results in (C) were obtained after washing at low stringency (2×SSPE, 37°C), and results in (D) at higher strigency (0.5×SSPE, 37°C). Mismatched hybrids were stable in (C) but not in (D), as is evidenced by the lack of hybridization in the CL-a lane. (**E**) Results from a separate PCR experiment hybridized with the probe RI5-482RC.

shown). To detect any specific product, these DNA fragments were transfered onto a nitrocellulose filter and hybridized with an oligonucleotide sequence from the amplified region of the R-element (RI5-482RC, probe a in Figure 1). A distinct band of expected size was detected in products from anlagen DNA from all stages of development, but not in products from DNAs of vegetative cells or micronuclei. Two such examples are shown in Figure 2A and E. Altogether we have tested nine different anlagen DNA samples (from cells at 12, 14, 16, 18 and 24 h of mating); all consistently gave positive results, except for two samples (14AN-a and 18AN-a, Figure 2) that occasionally generated less specific products. The reason for this variation is not clear. The major products detected in these studies were similar in size. Occasionally a band of greater mobility was also detected, which appeared to be the single-stranded form of the major product, since heat denaturation caused both products to migrate as a single band at this position (data not shown).

These specific products were not detected in two micronuclear DNA samples and one whole cell DNA sample isolated from vegetatively growing cells (Figure 2). Vegetative cell DNAs from three additional strains were also tested and gave no specific products (data not shown). Thus these products are specific to developing anlagen. Because of their predicted size and stage-specificity, we conclude that these PCR products are derived from circular forms of the excised R-element.

Sequences of the R-element circle junctions

To further analyze the structure of these putative excised R-elements, we cloned the PCR products from a 16 and an 18 h anlagen sample. The desired clones were identified by colony hybridization using the R-element oligonucleotide probe (RI5-482RC). Five positive-hybridizing clones were analyzed, of which four had an insert of the expected size (one from the

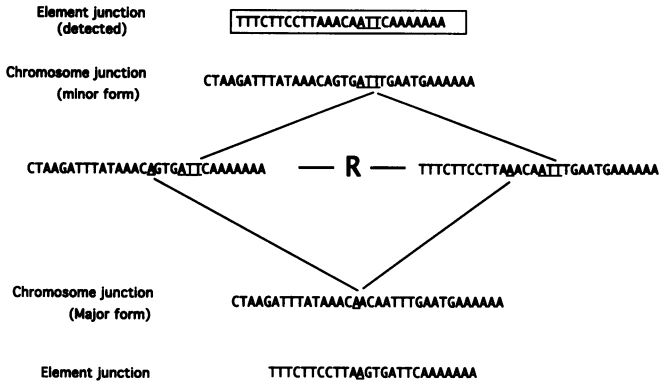

Figure 3. Junction sequences resulting from R-element deletion. Sequences surrounding the two ends of the R-element are presented in the middle of the figure. The sequences of a minor and a major form of the deletion junction remaining in the macronuclear DNA (chromosome junctions) are shown here above and below, respectively, the sequence of the element. The underlined nucleotides are the terminal repeats of the deletion elements, one copy of which is removed during deletion. Above and below these chromosome junctions are the corresponding element junctions expected if reciprocal exchange occurs during the deletion process. The element junction detected by cloning and sequencing in this study is shown at the top. It is enclosed by a box and represents the major element junction. The element junction shown at the bottom of the figure is a minor form detected only by hybridization.

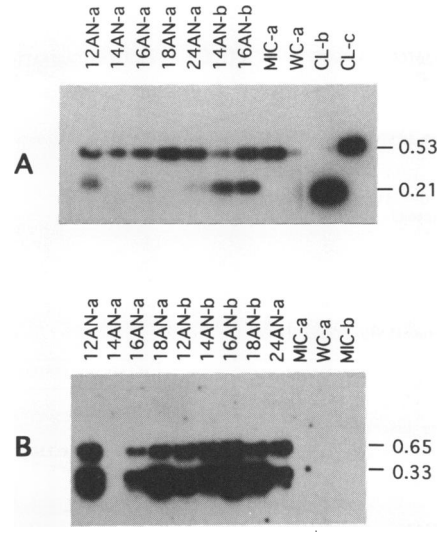

Figure 4. Detection of the excised M-elements by PCR. *Tetrahymena* DNAs were used as templates to detect the junction of the circular excised M-elements by PCR. About 5 μl of PCR products generated were analyzed by electrophoresis in a 1.5 % agarose gel, followed by blotting and hybridization with the oligonucleotide probe M1022 (probe b in Figure 1). Macronuclear anlagen, vegetative cell and micronuclear DNA were used as templates as described in Figure 2 and are indicated at the top of each lane. CL-b and CL-c are two control plasmids containing element junctions of a 0.6 and a 0.9 kb M-element derived from an earlier PCR experiment. They were digested with *Hin*dIII and *Eco*RI to separate the inserts from the vectors. Approximately 0.1 μg of these DNAs were loaded in these two lanes. (A, B) The results from two independent PCR experiments, using two different sets of primers: Primers 3 and 4 were used in (**A**), and primers 5 and 6 in (**B**). CL-a and CL-b lanes showed intense hybridization, and were blocked from the intensifying screen during this exposure to prevent darkening of the neighboring lanes.

18 h sample and three from the 16 h sample). They contained identical R-element sequence extending outward from the two PCR primer sequences, which were joined at the two ends of the element to form a circle junction (Figure 3). This junction sequence is one of the two predicted from the junction sequences known to be present in the macronuclear chromosome, if deletion involves a precise reciprocal exchange (Figure 3, and below). The fifth clone had a shorter insert and contained only one of the two PCR primer sequences. It is believed to be derived from a broken fragment or an aborted PCR product.

In earlier studies we have found two versions of the junction sequences retained in the macronuclear genome resulting from R-element deletion (referred to as chromosome junctions). One is present in the vast majority of mature lines (95% or more) examined and the other in very few of them (6, 12). This biased distribution also exists in DNA of developing macronuclei. We amplified this region from two anlagen DNA samples (14AN-a and 16AN-a) by PCR and determined their sequences directly without cloning. In both cases the predominant sequence was identical to the major type, but a minor component agreeing with the minor type described above was also detected (data not shown).

Based on these two chromosome junction sequences one can predict two junctions for the excised elements (referred to as element junctions) if deletion occurs through precise reciprocal exchange (Figure 3). Surprisingly, the element junction detected in this study corresponds to the minor chromosome junction. To rule out cloning bias, PCR products were hybridized with oligonucleotide probes which match the two predicted element junctions (Figure 2B–D). Probe JRE1, which contains the observed element junction, hybridized strongly with the specific PCR products from all anlagen templates under stringent conditions, indicating the presence of an identical or nearly identical sequence in these samples. On the other hand, JRE2, which contains the other predicted element junction sequence, hybridized poorly to only two of the PCR samples analyzed (Figure 2D). This consistant difference suggests a significant difference in the abundance of these two sequences in the cell. This result confirms that the major element junction sequence is the one predicted from the minor chromosome junction sequence. In addition, the element junction corresponding to the major chromosome junction is also present as a minor form in these developing cells.

Deletion products of the M-element

To determine whether other deletion elements in *Tetrahymena* also form covalent circles, we analyzed the neighboring M-element by a similar method. Two alternative forms (a 0.6 and a 0.9 kb form) of this element are deleted during macronuclear development (Figure 1). We chose two divergently oriented oligonucleotide sequences from within the 0.6 kb deleted region to serve as primers (M619R and M1001, primers 3 and 4 respectively in Figure 1), and DNA isolated from developing macronuclear anlagen, vegetative cells and micronuclei as PCR templates. Similar to the results of the R-element analysis, numerous DNA fragments of various sizes were generated, and products specific to the M-element were identified only after hybridization with an M-element probe, oligonucleotide M1022 (probe b in Figure 1). This probe should detect PCR products corresponding to both the 0.6 and the 0.9 kb deletion, with expected sizes of 210 and 525 bp respectively. Figure 4 shows

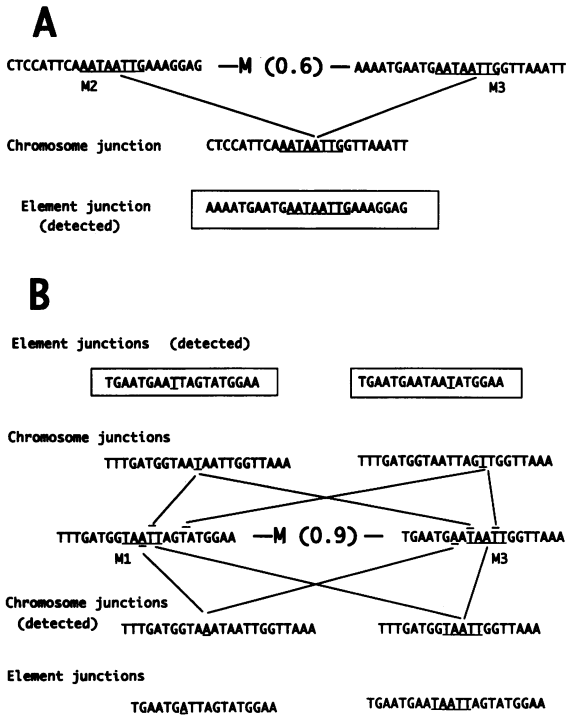

Figure 5. Junction sequences of the M-elements. Junction sequences of the 0.6 and the 0.9 kb M-element are shown in the upper and lower halves of this figure respectively. In each case the junction sequence of the circularized element (element junctions) is shown above or below the corresponding junction sequences retained in the chromosome (chromosome junction), and are shown next to the sequence of the elements. The underlined nucleotides mark the boundaries of each deletion event. They are terminal direct repeats of 1–8 bp in length. The element junctions detected in this study are enclosed in boxes. For the 0.6 kb form, both the element and chromosome junctions shown here have been detected. For the 0.9 kb deletions, only the chromosome junctions shown below the element sequence and the element junctions shown above it have been detected.

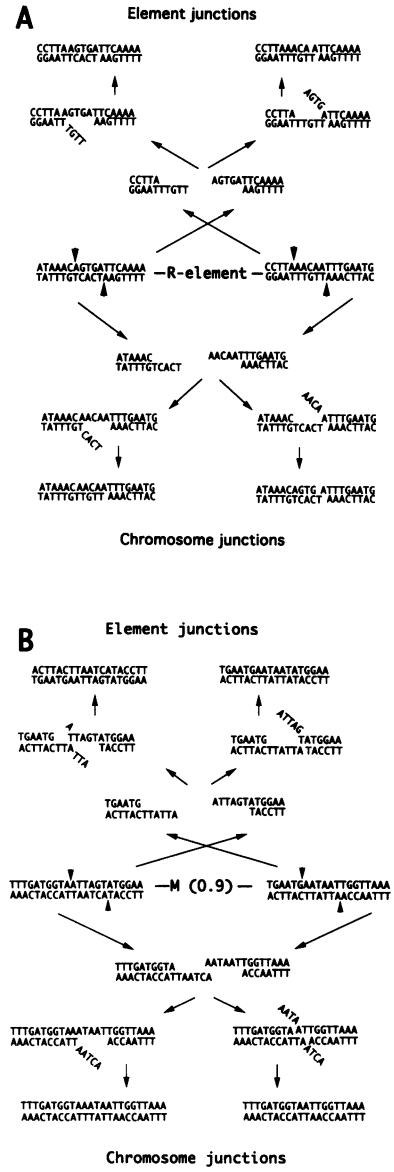

Figure 6. A model for the formation of deletion junctions. In this model staggered cuts occur at both ends of the deletion element. The ends generated are joined after repair to form the junctions detected. This model is illustrated with the deletion of the R-element in (**A**), and the 0.9 kb M-element in (**B**). The R-element is excised by a pair of staggered cuts to generate single-stranded protruding ends which are five or more nucleotides in length, and are different in sequence for the two ends. These ends are joined following mismatch repair. Depending on which strand is used as a template and which strand is degraded, different junction sequences are created. A similar process also occurs for the 0.9 kb M-element, except that in this case the staggered cuts generate protruding ends of six or more nucleotides in length. In addition, both protruding ends can be degraded partially during joining and mismatch repair. Deletion of the 0.6 kb M-element can also be explained by this model, but is not illustrated here. This model explains all of the junctions detected, and predicts the presence of other junctions with sequence variations in the regions covered by the staggered cuts.

that two bands of the expected sizes are detected in DNA samples from developing anlagen. The faster migrating species was not detected in DNAs from vegetative cells or micronuclei, and is thus a developing cell-specific product. Little or no product was generated from one of the 14 and one of the 18 h samples in some trials. The reason is unclear, but probably reflects the low abundance of circle junctions in these samples. The slower migrating band is also detected in DNAs from vegetative cells. It probably contains other DNA in addition to the excised 0.9 kb M-element. The identity of these other products is not clear. To further clarify this point we used a different pair of primers (M601R and K861M, primers 5 and 6 in Figure 1) which are located within the 0.6 kb element for PCR. Both the 0.6 and 0.9 kb excised forms are detected in all but one of the anlagen DNAs tested (Figure 4B). A third band with a greater mobility was also detected occasionally, and is probably the single-stranded form of the 0.6 kb element product. In contrast, none of these products were detected in DNA samples from vegetative cells, including two micronuclear DNA and one whole cell DNA preparations. Three additional vegetative cell DNAs have also been tested with similar results (data not shown). Thus, similar to the R-element, deletion of both the 0.6 and 0.9 kb M-element also produces circular exised forms.

Sequences of the M-element circle junctions

To further study these excised elements, we cloned the PCR products from a 12 and a 16 h anlagen sample obtained using the first set of primers. A total of five clones that hybridized with the oligonucleotide M1022 were analyzed, of which two

contained inserts of the size expected for the 0.6 kb element, and had the expected sequence for the excised form including one copy of the 8 bp terminal direct repeat at the circle junction (Figure 5). The previously determined chromosome junction sequence also retains one copy of the 8 bp repeat, and is invariant among more than 20 independent lines analyzed (12). The other three clones contained larger inserts. They all contained the same junction sequence as the first two clones analyzed above plus sequences of unknown origin, and are apparently derived from multiple fragment ligation during cloning. Thus all five clones analyzed here contain identical junction sequences.

None of the clones analyzed above contained fragments corresponding to the 0.9 kb deletion. To identify these clones an oligonucleotide (M299RC, probe c in Figure 1) from the region between M1 and M2 was used as a probe in colony hybridization. Four clones were identified, all containing inserts of the size expected for the 0.9 kb deletion. Sequence studies confirmed their expected structure and revealed two junction sequences, one in three clones and the other in the fourth (Figure 5B, boxed sequences). Previous studies have revealed two junction sequences in the macronuclear chromosome for this deletion: one contains a copy of the 5 bp terminal repeat, and the other differs from it by 4 bp in this region (Figure 5B) (12). Other chromosome junctions must also exist, since some lines contain neither of these sequences. The two element junctions obtained here are not the expected reciprocal exchange products of these two chromosome junctions. Together with the R-element results, these results raise interesting questions about the mechanism of DNA deletion. A model is presented in the next section to explain these results.

DISCUSSION

Using PCR we have detected DNA sequences resulting from the joining between the two ends of the deletion elements R and M in *Tetrahymena*, suggesting that some of these elements are circularized during excision. We believe these products are not artifacts of PCR for the following reasons. First, they are detected only in conjugating cells when DNA deletion occurs. They are found in 9 different macronuclear anlagen DNA samples from cells at 5 different stages of conjugation, and not in DNA samples from vegetatively growing cells, including whole cell DNA from four different strains and micronuclear DNA from two different strains. Micronuclear DNA provides a good comparison, since it contains similar amounts of deletion elements as the early anlagen DNA does. Second, the circle junctions detected have the structure expected from a simple deletion process. Although PCR could generate artificial recombinants between templates with stretches of sequence identity, it is highly unlikely that this process generates the products reported here. This point is particularly evident in the cases of the R-element and the 0.9 kb M-element, in which the circle junctions occur at positions with only 1−3 bp of sequence identity, which clearly are not among the most likely sites for PCR-derived recombinations.

The excised circles apparently are present in very low abundance. They have not been detected previously in purified anlagen DNA by genomic DNA hybridization methods (3). The difficulty with which these structures were detected by PCR in this study further supports this conclusion. In all cases the specific products were detectable only after Southern hybridization, and in some of these cases they were not consistently detected. This low abundance suggests that the excised circles are either very unstable or formed only rarely, or both. We have detected the circle junctions in cells as late as 24 h after mating begins, whereas the M- and R-element are known to be deleted at a much earlier stage (between 12 and 14 h after conjugation begins) (3). Thus the circles are probably quite stable once formed, and are lost only after the cell finishes conjugation and begins to divide. To account for their low abundance, we suggest that only a small proportion of the excised elements form stable circles. We think that the elements are excised primarily as a unstable form, from which the stable circles are formed occasionally. The unstable form could be linear, non-covalently joined circlular, or circular molecules destined for rapid degradation due to their association with other cellular components. This interpretation implies that the stable circles detected here are not obligatory deletion intermediates. Nonetheless, their junctions are likely to reflect the ends of the primary excision products and shed light on the process of deletion.

The low abundance of the excised circles in *Tetrahymena* is different from the situations in *Euplotes* and *Oxytricha*. In these organisms the excised circles are quite abundant, and readily detectable among whole genomic DNA by ethidium bromide staining or by Southern hybridization after gel electrophoresis (15, 19, 26). This is a significant difference, which indicates that the formation of stable circles is not a necessary common step of DNA deletion in ciliates.

Although the stable excised circles may not be obligatory intermediates in DNA deletion, their junction structures could still provide useful information regarding the ends of the excised elements, whether they are linear or circular. These junctions are quite different from those in *Euplotes*, where extra flanking sequences are present. No extra bases are detected in *Tetrahymena*. Instead, two of the three junctions analyzed here show sequence micro-heterogeneity. In addition, these sequences cannot be explained by a simple reciprocal exchange when compared with the known chromosome junctions. We propose the following model to explain this observation. The R-element is excised by a pair of staggered cuts to produce termini with protruding ends that are not complementary in sequence (Figure 6A). Joining between the two chromosome ends or the two element ends is accompanied by repair synthesis. Depending on which strand is used as a template, either the major or the minor junction sequence is produced, and this can be different for the element and the chromosome junctions. For the 0.9 kb M-element, protruding ends with non-complementary sequences are also generated by staggered cuts. However, in this case both ends are degraded partially before copying and rejoining occurs (Figure 6B). This process could produce many possible junction sequences, including the four observed, which differ from one another only within the region covered by the staggered cuts. For the 0.6 kb M-element, the staggered cuts may occur within the 8 bp terminal repeats, such that homogeneous junctions are generated.

An important aspect of this model is that it also explains how micro-heterogeneity is created in the chromosome junctions. According to this model, the micro-heterogeneity is generated in both the element and chromosome junctions as a result of variation in repair synthesis, and not variation in cut sites. It also predicts a specific role for the terminal direct repeat in generating homogeneous junctions. If the staggered cuts occur entirely within the terminal repeat, the protruding ends produced should be complementary in sequence, leading to the formation of homogeneous junctions. Deletion of the 0.6 kb M-element

illustrates this point nicely. The deletion end points occur at a pair of 8 bp terminal repeats. Little or no heterogeneity has been detected among the five element junctions analyzed here or the more than 20 chromosome junctions analyzed previously. In cases where terminal repeats are shorter than the protruding ends generated, mismatch repair is required and junctional heterogeneity can be generated. The R-element and the 0.9 kb M-element illustrate these cases well, as described above. The model also predicts that the heterogeneous region will be confined to within the length of the protruding ends generated, and that no mosaic or new sequences will be created in the junction. This is generally true, but since the actual lengths of the protruding ends are not known, the exact boundaries of the heterogeneous region cannot be predicted. We have proposed five and six nucleotides for the lengths of the R- and the 0.9 kb M-element protruding ends in order to explain the data in hand. Their actual values could be larger. Although 5′ protruding ends are indicated in the figures, 3′ protruding ends are also consistent with these results.

This model can be tested by removing the terminal repeat and determining whether heterogeneous junctions are generated. This has actually been done in our recent studies of the M-element, in which the terminal repeat at one boundary (M3) is partially destroyed and the flanking polypurine signal is moved to three new sites. Deletions occur in all three constructs, and their right boundaries are at new sites determined by the polypurine signal, which lack obvious terminal direct repeats. In all three cases micro-heterogeneity is detected among the chromosome junction sequences analyzed (11).

In many ways this model is similar to the one proposed earlier to explain the deletion of two IES (internal eliminated sequence) and the Tec1 element in *Euplotes* (16, 17), which also involves staggered cuts and repair synthesis. However, in that model the joining and repair synthesis of the chromosome and the element junctions follows two invariable pathways. The model proposed here calls for flexible repair synthesis to explain the junction micro-heterogeneity detected in *Tetrahymena*. It can also explain the results of TBE1 excision in *Oxytricha* in much the same way as it explains the results of the 0.6 kb M-element. In this sense the model provides the argument that DNA deletions in ciliates, despite their apparent differences in junction structure, may share a common feature involving fixed staggered cuts followed by joining with possible mismatch repair. The lengths of the terminal repeats and protruding ends, the extent of repair and the choice of templates determine the exact junction structure produced. These four aspects of the deletion process could vary among different elements in different organisms. This explanation simplifies our view of the mechanisms of DNA deletion in ciliates.

While this study was being completed, we became aware of a similar study by Saveliev and Cox (27), who also used PCR to detect the excised circle junctions of the M- and the R-elements in conjugating cells. They have detected similar sequences and reached the similar conclusions that these circles are formed during the process of deletion, that they are stable but low in abundance, and that they are probably not obligatory intermediates of the process. In addition, they have detected a different class of circles that are mostly larger and quite variable in size, and contain sequences from one or both flanking regions of the elements. These larger circles are not detected in the studies reported here or in our unreported results, and thus are not supported, nor disproven, by our data. The reason for this difference is not clear. One possibility could be the use of whole cell DNA as PCR templates in their experiments, which contained large amounts of old macronuclear DNA and proportionally less amounts of developing macronuclear DNA. The higher sensitivity that was required to detect the circles in their study might have helped in bringing out rare and unusal products.

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REFERENCES

1. Yao, M.-C. (1989) Mobile DNA, Berg and Howe (eds). American Society for Microbiology. Washington, DC.
2. Yao, M. C., J. Choi, S. Yokoyama, C. F. Austerberry and C. H. Yao. (1984) Cell. **36**: 433–40.
3. Austerberry, C. F., C. D. Allis and M. C. Yao. (1984) Proc Natl Acad Sci USA. **81**: 7383–7.
4. Karrer, K. M. (1983) Mol Cell Biol. **3**: 1909–1919.
5. Yao, M. C. (1982) J Cell Biol. **92**: 783–9.
6. Austerberry, C. F. and M. C. Yao. (1987) Mol Cell Biol. **7**: 435–43.
7. Austerberry, C. F. and M. C. Yao. (1988) Mol Cell Biol. **8**: 3947–50.
8. Katoh, M., M. Hirono, T. Takemasa, M. Kimura and Y. Watanabe. (1993) Nucleic Acids Res. **21**: 2409–2414.
9. Heinonen, T. Y. and R. E. Pearlman. (1994) J Biol Chem. **269**: 17428–33.
10. Wells, J. M., J. L. Ellingson, D. M. Catt, P. J. Berger and K. M. Karrer. (1994) Mol Cell Biol. **14**: 5939–49.
11. Godiska, R., C. James and M.-C. Yao. (1993) Genes Dev. **7**: 2357–2365.
12. Austerberry, C. F., R. O. Snyder and M. C. Yao. (1989) Nucleic Acids Res. **17**: 7263–72.
13. Godiska, R. and M. C. Yao. (1990) Cell. **61**: 1237–1246.
14. Ribas-Aparicio, R. M., J. J. Sparkowski, A. E. Proulx, J. D. Mitchell and L. A. Klobutcher. (1987) **1**: 323–336.
15. Jahn, C. L., M. F. Krikau and S. Shyman. (1989) Cell. **59**: 1009–1018.
16. Jaraczewski, J. W. and C. L. Jahn. (1993) Genes Dev. **7**: 95–105.
17. Klobutcher, L. A., L. R. Turner and J. LaPlante. (1993) Genes Dev. **7**: 84–94.
18. Hunter, D. J., K. Williams, S. Cartinhour and G. Herrick. (1989) Genes Dev. **3**: 2101–12.
19. Williams, K., T. G. Doak and G. Herrick. (1993) EMBO J. **12**: 4593–4601.
20. Yokoyama, R. W. and M. C. Yao. (1982) Chromosoma. **85**: 11–22.
21. Gorovsky, M. A. (1973) J Protozool. **20**: 19–25.
22. Bruns, P. J. and T. B. Brussard. (1974) J Exp. Zool. **188**: 337–344.
23. Allis, C. D. and D. K. Dennison. (1982) Dev. Biol. **93**: 519–533.
24. Yao, M. C. and M. A. Gorovsky. (1974) Chromosoma. **48**: 1–18.
25. Sambrook, J., E. T. Fritsch and T. Maniatis. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. Tausta, S. L. and L. A. Klobutcher. (1989) Cell. **59**: 1019–26.
27. Saveliev, S. V. and M. M. Cox (1994) Nucleic Acids Res. **22**, 5695–5701.