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An Intramolecular Recombination Mechanism for the Formation of the rRNA Gene Palindrome of *Tetrahymena thermophila*

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Large palindromic DNAs are found in a wide variety of eukaryotic cells. In *Tetrahymena thermophila*, a large palindrome is formed from a single rRNA gene (rDNA) during nuclear differentiation. We present evidence that a key step in the formation of the rDNA palindrome of *T. thermophila* involves homologous intramolecular recombination. Heteroduplex micronuclear rDNA molecules were constructed in vitro and microinjected into developing macronuclei, where they formed palindromes. Analysis of the resulting palindromes indicated that both strands of the microinjected rDNA are used to form the same palindrome. This study, together with a previous study (L. F. Yasuda and M.-C. Yao, Cell 67:505–516, 1991), is the first to define a molecular pathway of palindrome formation. The process is initiated by chromosome breakage at sites flanking the micronuclear rDNA. An intramolecular recombination reaction, guided by a pair of short inverted repeats located at the 5′ end of the excised rDNA, covalently joins the two strands of micronuclear rDNA in a giant hairpin molecule. Bidirectional DNA replication converts the giant hairpin molecule to a palindrome. We suggest that the general features of this pathway are applicable to palindrome formation in other cell types.

The amplification of certain genes, either as a normal part of development or as a rare abnormal event in cell growth, is widespread in eukaryotes. Frequently, the chromosomal domain involved in amplification is organized as a large inverted duplication, or palindrome (see reference 10 for a review). Notable examples include the rRNA genes (rDNA) of several genera (*Tetrahymena*, *Dictyostelium*, and *Physarum*), some amplified oncogenes in tumor cell lines, and the genes conferring resistance to cytotoxic drugs in cultured mammalian cells, parasitic protozoa, and *Saccharomyces cerevisiae* (3, 11, 41). It has been suggested that palindrome formation is an initiating event of gene amplification, and because of its apparently seminal role, several complicated models describing the formation of palindromes have been put forth (6, 10, 15, 21, 22, 29, 31, 34, 39). However, it has been difficult to directly test these models in most systems because of the rarity of the relevant events. Thus, there is little or no hard evidence bearing on the question of how palindromic DNA structures are formed in eukaryotic cells.

The ciliated protozoan *Tetrahymena thermophila* offers a unique opportunity to dissect the molecular mechanism of palindrome formation. *T. thermophila* combines two main advantages over other experimental systems for this purpose. First, palindromes are formed in every cell as a regular part of nuclear differentiation (8, 17). During the vegetative stage of the life cycle, *T. thermophila* cells harbor two vastly different nuclei: a conventional diploid nucleus, called the micronucleus, and a highly polyploid nucleus, called the macronucleus. When two cells conjugate, *T. thermophila* enters a developmental phase, during which the old macronucleus is destroyed and a new macronucleus develops from a zygotic product of the micronucleus. During macronuclear development, the micronuclear genome is extensively reorganized by a number of processes, including site-specific chromosome breakage, new telomere synthesis, DNA deletion and splicing, gene amplification, and palindrome formation (see reference 45 for a review). Many of these processes, including palindrome formation, are exemplified by the rRNA gene (rDNA) (Fig. 1). In the micronucleus, a single copy of the rDNA is embedded in each of two homologous micronuclear chromosomes. The coding and regulatory sequences comprise approximately 10 kbp of chromosomal sequence. Flanking the rDNA are conserved 15-bp elements called Cbs, for chromosome breakage sequence (47). During macronuclear development, the rDNA is excised from its micronuclear chromosome by breakage at the flanking Cbs elements. Following excision, a new telomere is synthesized at the 3′ end of the molecule. At the 5′ end of the molecule, an undefined set of reactions result in the formation of a 20-kbp head-to-head dimer, or palindrome. The rDNA palindrome is subsequently amplified to approximately 10,000 copies per cell.

The second advantage of this system is that *T. thermophila* cells can be transformed by microinjection of rDNA (40, 46). If cloned micronuclear rDNA is successfully injected into developing macronuclei, it will undergo all of the processing steps described above to yield mature palindromic rDNA. The rDNA allele used for microinjection confers resistance to the antibiotic paromomycin. Thus, those cells that have taken up and processed the injected rDNA can be easily selected for further analysis.

The transformation system, coupled with in vitro mutagenesis of micronuclear rDNA clones, has proven to be a powerful approach to identifying the important *cis*-acting components signaling palindrome formation (48). At the extreme 5′ end of the micronuclear rDNA locus is a pair of 42-bp perfect inverted repeats separated by a 28-bp nonpalindromic spacer. Significantly, these inverted repeats are also at the very center of macronuclear palindromic rDNA (see Fig. 1). Deletion and insertion experiments demonstrated that the inverted repeats are absolutely required for the palindrome formation process. Furthermore, it was found that the symmetry of the inverted repeats, and not the specific sequence, is required for palin-

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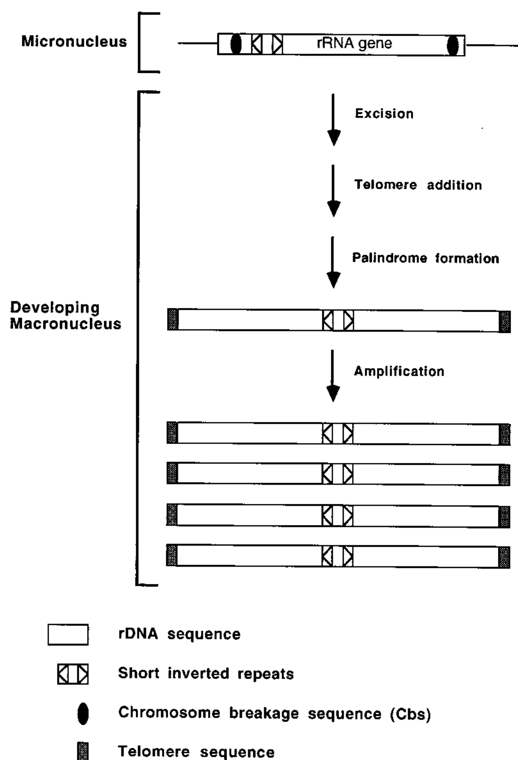


FIG. 1. Schematic diagram of rDNA processing events during macronuclear development. The top of the figure represents the micronuclear form of rDNA. The processing steps are as follows: excision of the rDNA from its micronuclear chromosome by breakage at the flanking Cbs sites; new telomere addition at the 3′ end of the excised rDNA; palindrome formation; and amplification of the palindromic rDNA to approximately 10,000 copies per macronucleus. The thin line represents micronuclear chromosomal sequence. Symmetrical triangles represent the inverted repeats plus spacer.

drome formation. This is an important result because it implies that intrastrand pairing of the inverted repeats is a necessary step in making the palindrome. Another essential feature of the process is that the inverted repeats must be adjacent to a chromosome end. The chromosome end is supplied in vivo by breakage at the Cbs located approximately 15 bp upstream of the inverted repeats. Finally, in coinjection experiments, it was found that palindromes are formed from single rDNA molecules and not from the joining of two rDNA molecules, indicating that a novel intramolecular process is involved (48).

The foregoing results clarify some important aspects of the palindrome formation process, namely, that a stem-loop structure formed by intrastrand pairing of the inverted repeats is present at the 5′ end of the excised rDNA and that an intramolecular reaction leads from this structure to a palindrome. A critical unanswered question is what is the mechanism of the intramolecular reaction? In this paper, we present evidence that an intramolecular recombination reaction, presumably guided by the stem-loop structure, produces a giant rDNA hairpin molecule. The giant hairpin is converted to a complete palindrome by a round of bidirectional DNA replication. These studies provide the first detailed picture of palindrome formation in any organism.

MATERIALS AND METHODS

Cells and culture conditions. The *T. thermophila* strains used in this study were CU427, CU428, HC76, and HC81. All four strains are of the inbreeding line B. CU427 and CU428 were obtained from Peter Bruns. HC76 and HC81 were derived from CU427 and CU428, respectively, through one round of genomic exclusion mating that retains the same macronuclear and micronuclear genotypes (41a). Cell growth and mating conditions were as previously described (48).

Plasmids. The micronuclear rDNA clones D5-5MSR3′ and pD5H8 have all of the sequence information necessary to form palindromes during macronuclear development. D5-5MSR3′ has a 448-bp deletion in the 5′ nontranscribed spacer region. The deletion serves as a physical marker to distinguish injected rDNA from host rDNA (see reference 48 for details). This sequence is not essential for rDNA replication or rRNA expression. D5-5MSR3′ and pD5H8 each have a point mutation in the 17S coding region that confers resistance to the antibiotic paromomycin. Each plasmid has the C3 origin of replication. This origin gives the injected rDNA a replication advantage over the endogenous B type rDNA, facilitating complete replacement of the endogenous rDNA by the injected rDNA (30). D5-5MSR3′ has a polylinker a few bases downstream of the inverted repeats that contains unique sites for *Not*I, *Bst*EII, *Sma*I, and *Apa*I (see reference 48 for details). pD5H8 has a polylinker at position 8766 of the rDNA, with a unique *Not*I site (12).

Construction of heteroduplex rDNA and cloning. Annealing of single-stranded oligonucleotides was done as follows. Appropriate pairs of oligonucleotides were mixed in equal molar concentrations in 0.3 M sodium acetate, pH 5.2, boiled briefly, and allowed to cool to room temperature overnight. The annealed oligonucleotides were precipitated with 2 volumes of ethanol. The pellet was washed once in 70% ethanol and resuspended in TE (10 mM Tris [pH 8.0], 1 mM EDTA).

To construct the 5′ Het rDNA, D5-5MSR3′ was first digested with *Apa*I and *Bst*EII and then ligated with the *Dra*III-A/*Pvu*II-A or *Dra*III-B/*Pvu*II-B heteroduplex oligonucleotide. Ligations were carried out at a 1:1 molar ratio of double-stranded oligonucleotide to vector. To construct the 3′ Het rDNA, pD5H8 was first digested with *Not*I and then ligated with the *Apa*I-A/*Bst*EII-B heteroduplex oligonucleotide. Ligations were carried out at a 1:1 molar ratio of double-stranded oligonucleotide to vector. The heteroduplex oligonucleotides were phosphorylated prior to most ligations. In all cases, successful ligation destroys the restriction sites used to linearize the vector. Following all ligations, the DNA was digested with the restriction enzyme or enzymes used to prepare the vector for ligation. This step will linearize the nonrecombinant vector and render it incapable of transforming *T. thermophila*; vector-oligonucleotide recombinant molecules will remain intact. The DNA was then phenol-chloroform extracted once, chloroform extracted twice, precipitated with sodium acetate and ethanol, washed with 70% ethanol once, and resuspended in TE. Prior to microinjection, the DNA was centrifuged at 30,000 rpm for 20 min to pellet any impurities that might clog the injection needle.

Construction of the hairpin rDNA was done as follows. We synthesized a self-complementary oligonucleotide that is predicted to form a stem-loop structure. The sequence of the loop is the same as that at the very center of native rDNA. Embedded in the base-paired region is a C/C mismatch. The nine bases surrounding the mismatch are identical to that in the 5′ Het rDNA construct. Plasmid D5-5MSR3′ was digested with *Not*I and *Kpn*I to release the short inverted repeats and the 5′ Cbs on a small fragment. The digested vector was then ligated with the phosphorylated hairpin oligonucleotide at a molar ratio of oligonucleotide to vector of 100:1.

The pD5H8-*Apa*I and pD5H8-*Bst*EII clones were constructed by inserting the *Apa*I-A/*Bst*EII-B heteroduplex oligonucleotide into the *Not*I site of pD5H8, transforming *Escherichia coli*, and screening the transformants for plasmids that had acquired a new *Apa*I site or a new *Bst*EII site.

Microinjection. Microinjection of rDNA into developing macronuclei was carried out by established protocols (40, 46). Typically, 300 to 600 cells were injected per DNA sample. Following injection, cells were cloned in proteose-peptone medium and grown for 3 days. Clones were then replica plated to paromomycin-containing medium. Typically, paromomycin-resistant clones were apparent within 24 to 48 h after application of the drug. Transformation frequencies ranged from 1 to 5% except for the hairpin injection, when the transformation frequency was about 10-fold lower. Resistant cells were transferred to 10-ml cultures with paromomycin, grown for 2 to 3 days, and harvested for DNA isolation.

DNA analysis. *T. thermophila* DNA was isolated by established procedures (1). Restriction digestion, gel electrophoresis, Southern blotting, and probe hybridization were carried out by established protocols (48). Oligonucleotide probes were labeled with ^{32}P by using T4 polynucleotide kinase. Enzymes were purchased from New England Biolabs.

Oligonucleotides. The oligonucleotides used in heteroduplex experiments are listed below. Complementary pairs are indicated by the same uppercase letter following the name (e.g., *Dra*III-A is complementary to *Pvu*II-A). The oligonucleotides were: *Dra*III-A, 5′-GGTGATCACCTGGTGGTACC-3′; *Pvu*II-A, 5′-GTTACGGTACCACCAGCTGATCACCGGCC-3′; *Dra*III-B, 5′-GTTACGGTACCACCTGGTGATCACCGGCC-3′; *Pvu*II-B, 5′-GGTGATCACCAGCTGGTACC-3′; *Dra*III-C, 5′-GGCCGGCCGCCCGGGCACCTGGTGACC-3′; *Pvu*II-C, 5′-GGCCGGTCACCAGCTGCCCGGGCGGCC-3′; *Apa*I-A, 5′-GGCCGTGTGGGCCCGTAACCGTGT-3′; *Bst*EII-A, 5′-GGCCACACGGTTACCGGCCCACAC-3′; and hairpin oligonucleotide, 5′-GGCCGGCACCAGCTGAAGGCTAGTTTTTTTGCTTTTTGTTGTTAGTTTTATAGCCTTCACCTGGTGCC-3′. The following oligonucleotides were used as hybridization probes:

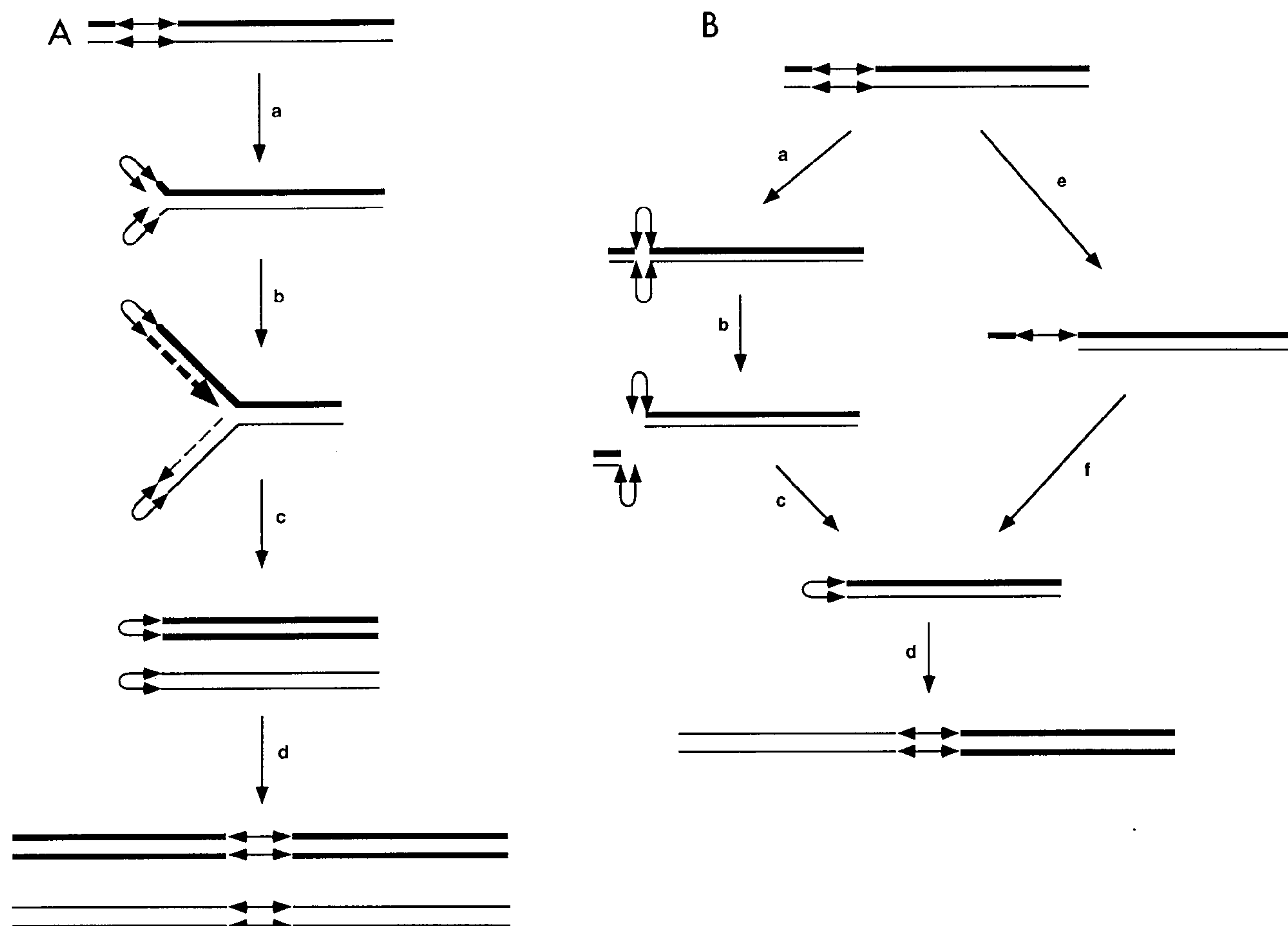


FIG. 2. Models of palindrome formation. The two strands of micronuclear rDNA are depicted as thick and thin lines. (A) Replication model. The model begins with excision of the rDNA. Step a, sequences upstream of the inverted repeats are eliminated, followed by intrastrand pairing of inverted repeats. Step b, beginning of DNA synthesis; leading-strand synthesis (thick dashed line) is primed at the base of the upper stem; lagging-strand synthesis (thin dashed line) is primed in the replication fork and proceeds toward the inverted repeat. Step c, replication is complete; the products are two giant hairpin molecules. Step d, bidirectional DNA replication originating within the giant hairpin forms the mature rDNA palindrome. (B) Intramolecular recombination model. The model begins with excised rDNA. Steps a, b, c, and d outline the cruciform cleavage pathway. Step a, intrastrand pairing of the inverted repeats. Step b, cleavage of the cruciform structure across the four-way junction by a resolvase. Step c, sealing of the nick by ligase to form a giant hairpin. Step d, bidirectional DNA replication originating within the giant hairpin forms the mature rDNA palindrome. Steps e, f, and d outline the SSA pathway. Step e, 5′-to-3′ exonuclease degrades one strand of the inverted repeats. Step f, self-annealing of the inverted repeat, excision of nonhomologous sequences, and sealing of the nick by ligase forms a giant hairpin. Step d, bidirectional DNA replication originating within the giant hairpin forms the mature rDNA palindrome. The thick line represents one of the original strands of micronuclear rDNA, and the thin line represents the other strand (see text). The double arrowheads represent the inverted repeats.

C3A, 5′-GCAACTTTTGAGACTTCGTG-3′; and MCA, 5′-AGGCTAGTTTT TTTGCTTTTTGTTGTTAGTTTTA-3′.

RESULTS

Models of palindrome formation. Two types of models have been proposed to explain how palindrome formation occurs in *T. thermophila* (48). Both models begin with chromosome breakage, so that the inverted repeats are adjacent to a chromosome end. The first model is based solely on DNA replication (Fig. 2A). According to this model, intrastrand pairing of the inverted repeats resembles a replication fork and, as such, is a substrate for replication factors (step a, Fig. 2A). Leading-strand synthesis is primed at the base of the stem with a free 3′ hydroxyl group, and lagging-strand synthesis is primed in the fork (step b). Complete replication in this fashion generates two giant hairpin molecules (step c). Each hairpin is converted to a palindrome by a round of bidirectional DNA replication, presumably originating at the known origin of replication upstream of the rRNA gene promoter (step d). There is precedent for this type of mechanism in the replication of adeno-associated virus and parvovirus genomes (7, 36).

The second model is based on intramolecular recombination (Fig. 2B). In this model, the intrastrand pairing of the inverted repeats is proposed to resemble a cruciform structure (step a). The cruciform can isomerize to a Holliday structure, an intermediate in homologous recombination. The concerted action of a nuclease that nicks two diagonally apposed strands at the four-way junction of the cruciform (step b) and a ligase to seal the nick will generate a giant hairpin molecule (step c). Nucleases—sometimes referred to as resolvases—that specifically recognize and cleave Holliday structures in this manner have been identified in *E. coli*, *Saccharomyces cerevisiae*, and mammalian cells (2, 14, 38, 42). As above, a round of bidirectional DNA synthesis originating within the giant hairpin forms the complete palindrome (step d). A second intramolecular recombination mechanism, based on the single-strand annealing

(SSA) model developed from work with mammalian cells, *Xenopus laevis*, and *S. cerevisiae*, will also form a giant hairpin (5, 9, 20). The essential features of this mechanism are drawn on the right side of Fig. 2B. After chromosome breakage at the 5′ Cbs, a 5′-to-3′ single-strand exonuclease creates a 3′ overhang encompassing the inverted repeats (step e). Self-annealing of the 3′ overhang (driven by the inverted repeat), excision of nonhomologous sequences, filling in of any gap, and ligation of the resulting nick create a giant hairpin molecule (step f). As above, a round of DNA synthesis converts the hairpin to a palindrome (step d).

The replication and intramolecular recombination models make different predictions about whether the two arms of a palindrome are derived from the same strand of micronuclear rDNA. In Fig. 2, we have coded one strand of micronuclear rDNA as thick and the other as thin so that their fates are easier to follow. Subsequent replication products from each strand are thick or thin according to which strand is used as a template. The distinguishing feature of the intramolecular recombination models is the covalent joining of the original strands of micronuclear rDNA in the giant hairpin molecule (steps c and f, Fig. 2B). Following replication of such a hairpin, one arm of the resulting palindrome is derived from one of the micronuclear rDNA strands, and the other arm of the same palindrome is derived from the other micronuclear rDNA strand.

Palindrome formation by the replication model is different. As is usually the case in DNA replication, the template strands segregate to different molecules following the completion of replication (step c, Fig. 2A). Each hairpin is derived from a different micronuclear rDNA strand. Therefore, in a palindrome produced by the replication mechanism, both arms of the same palindrome are derived from the same strand of micronuclear rDNA.

Heteroduplex experiment. The approach that we have taken to distinguish between the replication and recombination models is to uniquely mark each strand of a micronuclear rDNA plasmid in vitro and microinject the marked rDNA into developing macronuclei. The two strands of the rDNA were marked by inserting a heteroduplex oligonucleotide into the micronuclear rDNA clone D5-5MSR3′ (Fig. 3 and Materials and Methods). The heteroduplex oligonucleotide has a one-base mismatch (C/C), so that one strand encodes a recognition site for the restriction enzyme *Dra*III and the other strand encodes a recognition site for the restriction enzyme *Pvu*II. The site of insertion in D5-5MSR3′ is located a few bases downstream of the inverted repeats. We refer to the rDNA with the heteroduplex at this position as 5′ Het rDNA. The micronuclear rDNA clone D5-5MSR3′ has all of the sequence information necessary to form a palindrome during macronuclear development. The rDNA has a point mutation conferring resistance to the antibiotic paromomycin, which allows selection of cells transformed by D5-5MSR3′, and the replication origin of the C3 inbreeding line, which allows the injected rDNA to be preferentially maintained in the B inbreeding line host (30). Figure 3A depicts the 5′ Het rDNA molecule and the three types of palindromes that it can generate, depending on the mechanism of palindrome formation. The intramolecular recombination model predicts that a palindrome formed from the 5′ Het rDNA will be heterodimeric for the *Dra*III and *Pvu*II restriction sites; that is, a *Dra*III site on one arm and a *Pvu*II site on the other arm (Fig. 3A). The replication model predicts that a palindrome formed from the 5′ Het rDNA will be homodimeric for either the *Dra*III or *Pvu*II restriction site. That is, a given palindrome will have either a *Dra*III site on each arm or a *Pvu*II site on each arm (there are other plausible ways to generate homodimeric rDNA from this type of experiment, and these will be discussed in detail below).

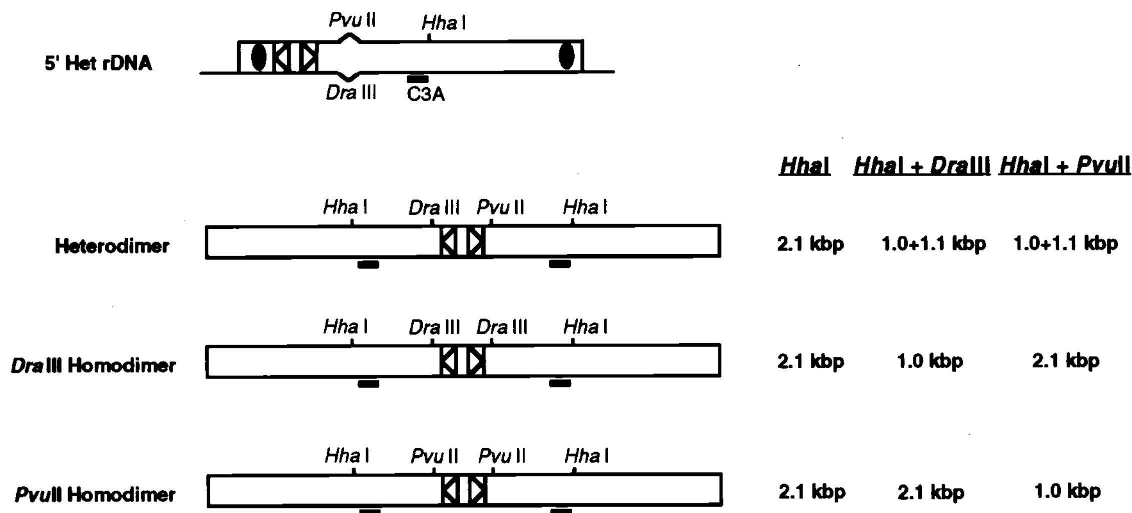
The 5′ Het rDNA was injected into developing macronuclei, and transformed lines were analyzed for the arrangement of *Dra*III and *Pvu*II sites on their rDNA. Genomic DNA from each transformant was doubly digested with *Hha*I and *Dra*III or *Hha*I and *Pvu*II. The digested DNAs were fractionated by agarose gel electrophoresis, blotted, and hybridized with a radiolabeled oligonucleotide (C3A) that is specific for the injected rDNA. Figure 3A shows the structures of the three possible palindromes and the expected sizes of the fragments that hybridize to C3A following the digestions. *Dra*III-*Pvu*II heterodimers will yield a 1.0-kbp fragment and 1.1-kbp fragment for each double digestion. The 1.1-kbp fragment spans the center of the palindrome and is diagnostic for the heterodimers. Homodimers will yield only the 1.0-kbp fragment or a 2.1-kbp fragment, depending on which site is homodimeric (see Fig. 3 for details).

Figure 3B shows an analysis of 10 typical transformants. Surprisingly, both heterodimers and homodimers were frequently observed. Of 31 transformed lines analyzed, 17 had clear evidence of the 1.1-kbp band indicative of heterodimeric rDNA and 28 showed evidence of homodimeric rDNA. Fourteen lines were mixtures of heterodimeric and homodimeric rDNA; the relative amount of each type of rDNA varied from transformant to transformant. When heterodimeric rDNA predominated, the homodimeric rDNA was evidenced by the presence of the 2.1-kbp band in one of the double digestion lanes. It is not surprising that several lines were mixtures, since hundreds of molecules are injected and usually more than one of these establishes the mature rDNA pool (37). Interestingly, when homodimers are present, they are usually of one or the other type and rarely both (the possible significance of this is discussed below). Occasionally the C3A probe detected a 2.0-kbp band (see transformant 6 in Fig. 3B). The origin of this band is unknown. Preliminary analysis indicates that the rDNA from which this fragment is derived lacks sequences located at the natural center of the rDNA palindrome and is therefore an aberrant product of transformation (data not shown).

The observation of heterodimeric rDNA is a robust result. The only way to generate heterodimeric rDNA from this type of experiment is by an intramolecular recombination mechanism. In theory, intermolecular recombination between a *Dra*III homodimer and a *Pvu*II homodimer in the small interval (150 bp) encompassing the inverted repeats could also generate heterodimeric rDNA. However, from coinjection experiments carried out previously, we know that the frequency of intermolecular recombination in the inverted repeats is so low as to be undetectable (48). Therefore, we can unequivocally conclude that intramolecular recombination is one pathway by which rDNA palindromes are formed in *T. thermophila.*

The SSA mechanism and the origin of homodimers. Homodimeric rDNA was a frequent outcome of the 5′ Het rDNA experiment. As shown in Fig. 2A, a replication-based mechanism of palindrome formation will produce homodimeric rDNA. However, the assumptions of the model predict that both types of homodimer will be formed in the same developing macronucleus. In our experiments, we rarely (only 1 of 31) recovered a transformant that had both types of homodimer. From the results of a coinjection experiment reported previously, there is no reason to assume that both cannot be maintained within the same macronucleus (48). Thus, we considered alternative explanations for the formation of homodimeric rDNA. One alternative involves the SSA model of recombination, and the second involves DNA mismatch repair.

A.



B.

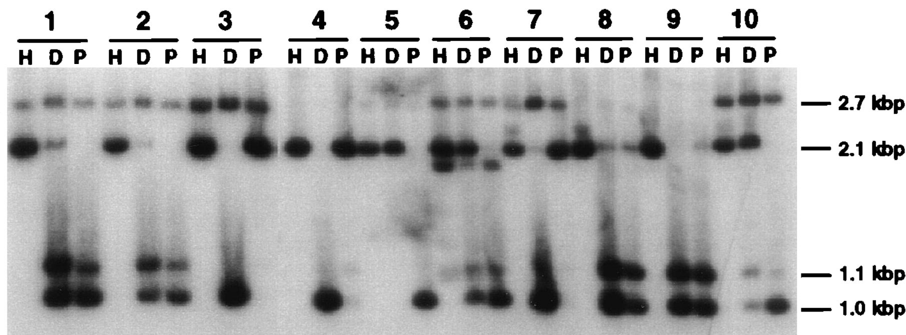


FIG. 3. 5′ Het rDNA experiment. (A) Schematic diagram of 5′ Het rDNA and the three types of rDNA palindromes that it can produce: *Dra*III-*Pvu*II heterodimer, *Dra*III homodimer, and *Pvu*II homodimer. The restriction enzymes used and the expected fragment sizes for each type of palindrome with the C3A oligonucleotide as a probe are given at the right. The position of the C3A probe is indicated for the 5′ Het rDNA and for each palindrome. Note that the probe hybridizes to each arm of a palindrome. Open rectangle, rDNA; thin line, vector sequence; solid oval, Cbs; symmetrical open triangles, inverted repeats. Carets in the 5′ Het rDNA represent the C/C mismatch. The short thick line is the C3A probe. The diagram is not to scale. (B) Southern blot analysis of 10 representative transformants from the 5′ Het rDNA microinjection. Transformants 4, 6, 7, 8, and 9 belong to set A, and transformants 1, 2, 3, 5, and 10 belong to set B (see text and Fig. 4 for details). Total genomic DNA (2 to 4 μg) from each transformant was digested with the indicated restriction enzymes, fractionated on a 1.5% agarose gel, blotted to a nylon filter, and hybridized with the radiolabeled C3A oligonucleotide. The 2.7-kbp band seen in some lanes is the result of intermolecular recombination between an injected rDNA palindrome and a host rDNA palindrome (48). This figure is a composite of two Southern blots (lanes 1 to 3 and lanes 4 to 10 are from separate blots). The heteroduplex oligonucleotides used in this experiment were not phosphorylated prior to ligation. H, *Hha*I; D, *Dra*III; P, *Pvu*II.

We have already described how the SSA mechanism of palindrome formation will produce heterodimeric rDNA (Fig. 2B). Figure 4 schematically shows how the SSA mechanism can also produce homodimeric rDNA in the context of the 5′ Het rDNA. An essential feature of the SSA model is a 5′-to-3′ exonuclease activity. If the 5′-to-3′ exonuclease reaches the heteroduplex region, then fill-in synthesis will eliminate the heteroduplex, and the resulting rDNA palindrome will necessarily be homodimeric. The C/C mismatch in the 5′ heteroduplex rDNA is only about 30 bp downstream of the inverted repeats, and thus it is conceivable that an exonuclease acting to generate the single-stranded protrusion might sometimes reach the mismatch. This is an attractive model, since it can explain the formation of heterodimers and homodimers.

The SSA model makes two testable predictions. The first prediction is that because of the 5′-to-3′ activity of the exonuclease, there will be a strand bias in the formation of homodimeric rDNA (see Fig. 4). In some of the 5′ Het rDNA molecules described above, the heteroduplex oligonucleotide was inserted in a known orientation. Thus, we know the polarity of the strand that encodes each restriction site. The type of homodimer that is produced (i.e., either *Dra*III or *Pvu*II) will tell us which strand of micronuclear rDNA is preserved during formation of the homodimer. In one injection experi-

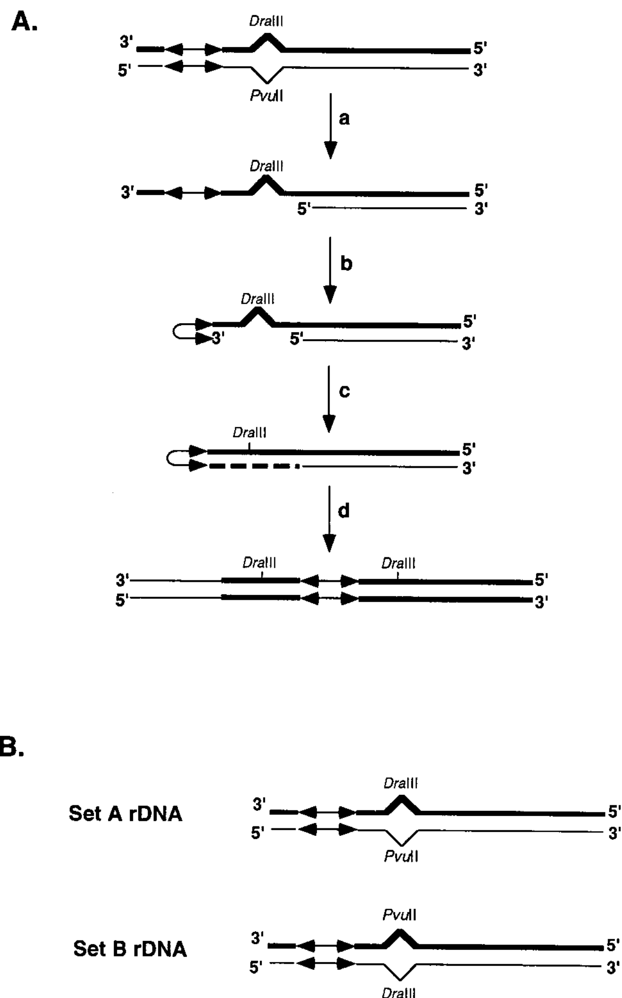


FIG. 4. (A) Schematic diagram depicting the formation of homodimeric rDNA by the SSA mechanism. At the top of the diagram is excised rDNA. The polarity of each strand is shown. As in Fig. 2, the two strands of micronuclear rDNA are depicted as thick and thin lines. Step a, the 5′-to-3′ exonuclease degrades past the C/C mismatch (carets). Step b, self-annealing of the inverted repeat and excision of nonhomologous sequences. Step c, DNA synthesis fills in the gap, eliminating the heteroduplex. Step d, the hairpin is replicated to form a homodimer. (B) Structures of the set A and set B rDNA molecules used for injection. The diagram is not to scale.

ment (set A), the *Dra*III site is encoded by the strand with its 3′ end oriented toward the upstream end of the rDNA. In a second injection experiment (set B), the *Pvu*II site is encoded by this strand. The SSA model predicts that homodimer-containing transformants in set A will be only of the *Dra*III type and that homodimer-containing transformants in set B will be only of the *Pvu*II type. Of 10 informative transformants in set A, 8 contained *Dra*III homodimers and 2 contained *Pvu*II homodimers. Of four informative transformants in set B, three had *Pvu*II homodimers and one had *Dra*III homodimers. While there appears to be a tendency to retain information from the strand with its 3′ end oriented toward the upstream end of the rDNA, it clearly does not happen all of the time.

A second prediction of the SSA model is that the greater the distance of the mismatch from the upstream end of the rDNA, the less likely it is that homodimeric rDNA will be produced. We have made a micronuclear rDNA molecule with a single-base mismatch located about 8.8 kbp from the upstream end of the rDNA (see Fig. 5A). This is in contrast to the 5′ Het rDNA, in which the mismatch was approximately 150 bp from the upstream end of the rDNA. We expect that a 5′-to-3′ exonuclease would be very unlikely, if ever, to reach a mismatch located 8.8 kbp from the upstream end of the rDNA.

The heteroduplex oligonucleotide used in this experiment has a C/C mismatch, so that one strand encodes a recognition site for *Apa*I and the other strand encodes a recognition site for *Bst*EII. These restriction enzymes were chosen because wild-type rDNA has no recognition sites for either *Apa*I or *Bst*EII. The heteroduplex oligonucleotide was inserted into a polylinker located 8,766 bp downstream of the inverted repeats. We refer to this rDNA as the 3′ Het rDNA. Figure 5A depicts the 3′ Het rDNA and the three possible types of palindromes that it can generate: an *Apa*I homodimer, a *Bst*EII homodimer, and an *Apa*I-*Bst*EII heterodimer. In *Apa*I homodimers, digestion with *Apa*I will give an approximately 16-kbp band detectable by the C3A oligonucleotide probe, whereas digestion with *Bst*EII will give full-length rDNA (approximately 21 kbp). The converse is true in *Bst*EII homodimers. In *Apa*I-*Bst*EII heterodimers, digestion with *Apa*I or *Bst*EII will give an approximately 18-kbp band (see Fig. 5A).

The 3′ Het rDNA was injected into developing macronuclei, 37 independent transformed lines were selected, and their rDNA was analyzed as described above. Both types of homodimers (*Apa*I and *Bst*EII) were frequently recovered. Homodimers were the predominant form of rDNA in approximately 50% of the transformants (20 of 37). Heterodimers accounted for another 20% (7 of 37). The remaining transformants displayed an approximately equal mix of homodimeric and heterodimeric rDNA. Figure 5B shows an analysis of 18 typical transformants.

Unlike the 5′ Het rDNA experiment, intermolecular recombination between palindromes in the mature macronucleus could be a potential confounding factor in interpreting these results. Since the two introduced restriction sites are separated by about 16 kbp, it is possible that the homodimers are the result of recombination between heterodimeric rDNA molecules. We cannot test for this type of recombination directly, but we can test for recombination between two different types of introduced homodimers to form a heterodimer. To do this, we coinjected two different rDNA plasmids marked at the site of the heteroduplex oligonucleotide insertion. One plasmid, pD5H8-*Apa*I, has a unique *Apa*I site in the 3′ polylinker, and the second plasmid, pD5H8-*Bst*EII, has a unique *Bst*EII site in the 3′ polylinker. They will produce only *Apa*I or *Bst*EII homodimers, respectively. Transformants from the pD5H8-*Apa*I and pD5H8-*Bst*EII coinjections were analyzed by *Apa*I and *Bst*EII digestions. The sizes of the heterodimer and homodimer bands are the same as those for the 3′ Het rDNA experiment. Figure 5C shows an analysis of nine representative transformants. The vast majority of rDNA in all of the transformants is homodimeric. In several lines, an 18-kbp band indicative of an *Apa*I-*Bst*EII heterodimer is visible. However, it invariably represents the minority of rDNA in the transformant. Therefore, intermolecular recombination occurs only rarely and cannot account for the production of homodimeric or heterodimeric rDNA detected in this study. From these results and the incomplete strand bias in the 5′ Het rDNA experiment, we conclude that the SSA mechanism is unlikely to account for the formation of homodimeric rDNA.

Mismatch repair and the origin of homodimers. A second explanation for the formation of homodimeric rDNA involves mismatch repair. For example, it is possible for homodimeric rDNA to be generated by intramolecular recombination if the mismatch is repaired prior to palindrome formation. To di-

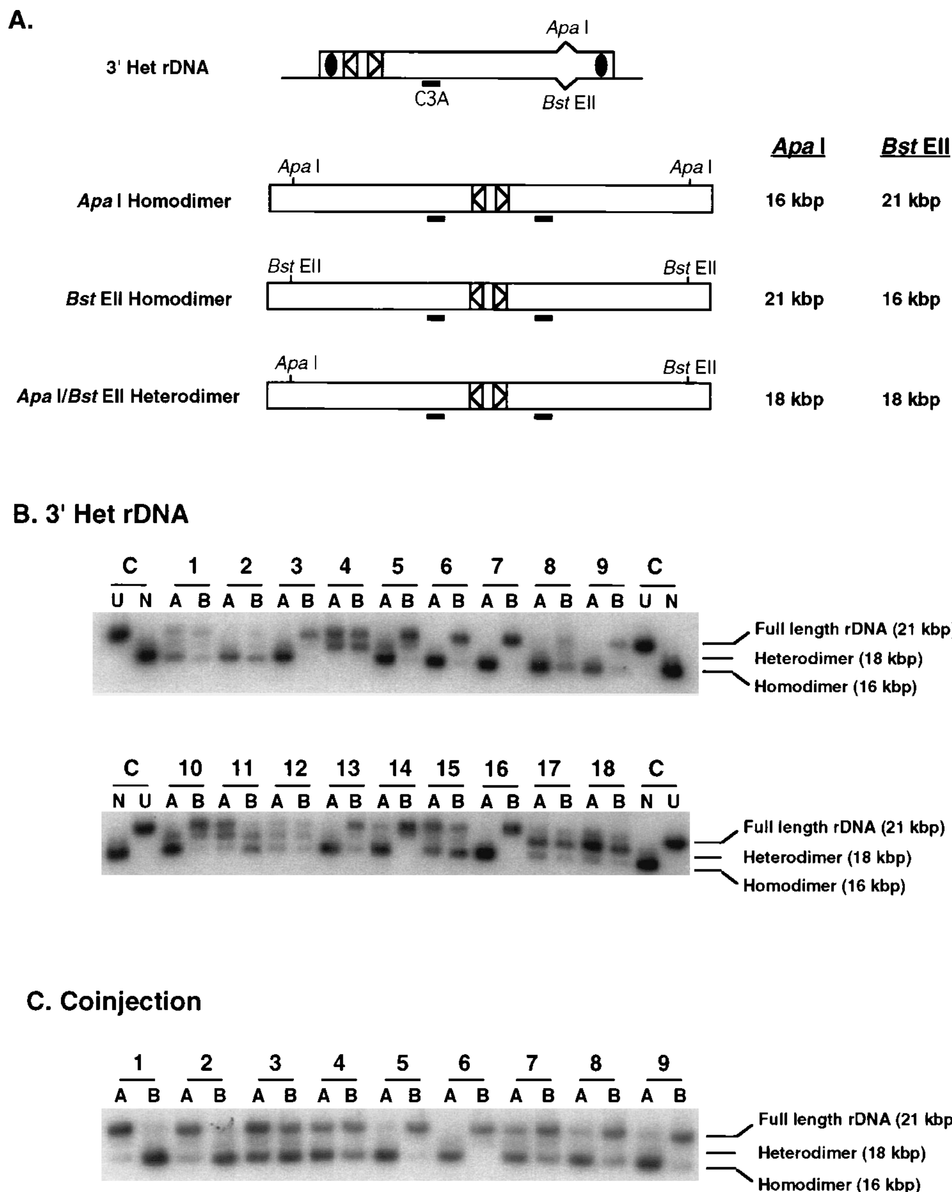


FIG. 5. 3′ Het rDNA experiment. (A) Schematic diagram of 3′ Het rDNA and the three types of rDNA palindromes that it can produce: *Apa*I homodimer, *Bst*EII homodimer, and *Apa*I-*Bst*EII heterodimer. The restriction enzymes used and the expected fragment sizes for each type of palindrome with the C3A oligonucleotide as a probe are given at the right. The position of the probe is indicated for the 3′ Het rDNA and for each palindrome. Open rectangle, rDNA; thin line, vector sequence; solid oval, Cbs; symmetrical open triangles, inverted repeats; carets in the 3′ Het rDNA, C/C mismatch; short thick line, C3A probe. The diagram is not to scale. (B) Southern blot analysis of 18 transformants from the 3′ Het rDNA microinjection. Total genomic DNA (2 to 4 μg) from each transformant was digested with the indicated restriction enzyme, fractionated on a 0.6% agarose gel, blotted to a nylon filter, and hybridized with the radiolabeled C3A oligonucleotide. Lanes C, control sample that is homodimeric for the restriction enzyme *Not*I. The position of the *Not*I site is the same as that for *Apa*I or *Bst*EII in homodimers. The heteroduplex oligonucleotides used in this experiment were phosphorylated prior to ligation. U, uncut; N, *Not*I; A, *Apa*I; B, *Bst*EII. (C) Southern blot analysis of nine transformants from coinjection of pD5H8-*Apa*I and pD5H8-*Bst*EII. Analysis and symbols are the same as in panel B.

rectly assess mismatch repair, we made an rDNA hairpin molecule containing a single C/C mismatch in vitro and injected this DNA directly into developing macronuclei (Fig. 6A). In this construct, the inverted repeats are deleted and the 5′ end of the rDNA is capped by a synthetic hairpin (see Materials and Methods). The rDNA has a Cbs at its 3′ end, so successful transformation is dependent on processing during macronuclear development. As in the 5′ Het rDNA experiment, the mismatch creates alternative recognition sites for *Dra*III and *Pvu*II. If the mismatch is repaired before the hairpin is replicated (i.e., before the hairpin is converted to a palindrome), then the palindrome will be either a *Dra*III homodimer or a *Pvu*II homodimer, depending on which strand is used as the template for repair. If the mismatch is not repaired, then the resulting palindrome will be a *Dra*III-*Pvu*II heterodimer (see Fig. 6A). The outcome (homodimer or heterodimer) is dependent only on whether or not the mismatch is repaired, not on the mechanism of palindrome formation.

The transformants were analyzed in the same way as those from the 5′ Het rDNA experiment except that the relevant fragments were detected on Southern blots by an oligonucleotide probe (MCA) that hybridizes to the central 34 bp of the

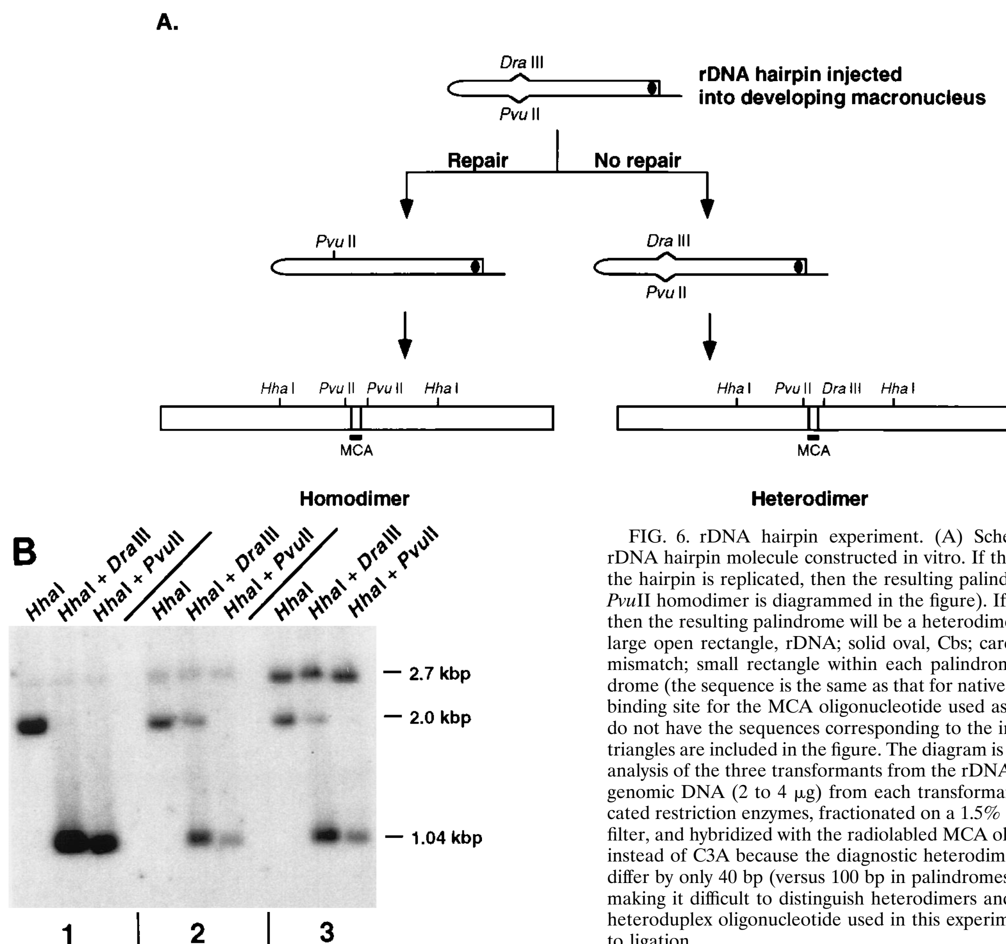


FIG. 6. rDNA hairpin experiment. (A) Schematic representation of the rDNA hairpin molecule constructed in vitro. If the mismatch is repaired before the hairpin is replicated, then the resulting palindrome will be a homodimer (a *Pvu*II homodimer is diagrammed in the figure). If the mismatch is not repaired, then the resulting palindrome will be a heterodimer. Thin line, vector sequence; large open rectangle, rDNA; solid oval, Cbs; carets in the hairpin rDNA, C/C mismatch; small rectangle within each palindrome, exact center of the palindrome (the sequence is the same as that for native rDNA palindromes and is the binding site for the MCA oligonucleotide used as a probe). These palindromes do not have the sequences corresponding to the inverted repeats, and hence no triangles are included in the figure. The diagram is not to scale. (B) Southern blot analysis of the three transformants from the rDNA hairpin microinjection. Total genomic DNA (2 to 4 μg) from each transformant was digested with the indicated restriction enzymes, fractionated on a 1.5% agarose gel, blotted to a nylon filter, and hybridized with the radiolabled MCA oligonucleotide. MCA was used instead of C3A because the diagnostic heterodimer band and the 1.0-kbp band differ by only 40 bp (versus 100 bp in palindromes derived from 5′ Het rDNA), making it difficult to distinguish heterodimers and homodimers with C3A. The heteroduplex oligonucleotide used in this experiment was phosphorylated prior to ligation.

rDNA palindrome. In heterodimers, MCA will detect only the 1.04-kbp fragment that spans the center of the palindrome. In homodimers, it will detect a 2.0-kbp band in one of the double digestion conditions.

For unknown reasons, the hairpin rDNA transformed poorly. Nevertheless, three transformants were obtained after repeated attempts. Figure 6B shows the analysis of these transformants. The first transformant has only heterodimeric rDNA. Double digestion with *Hha*I and *Dra*III or with *Hha*I and *Pvu*II gives only the 1.04-kbp band. Clearly, in this case the mismatch was not repaired. Significantly, the other two transformants do show evidence of mismatch repair. These transformants have some rDNA that is a *Pvu*II homodimer, as indicated by the 2.0-kbp band in the *Hha*I plus *Dra*III lane (Fig. 6B). This band will be present only if the C/C mismatch is repaired to a *Pvu*II site. These two transformants also have the 1.04-kbp band, indicative of heterodimeric rDNA. The fact that two of the three transformants have some homodimeric rDNA indicates that repair of C/C mismatches is a frequent event in developing macronuclei.

This experiment sheds light on another important issue. The final step of each model of palindrome formation is the conversion of a giant hairpin to a palindrome by bidirectional DNA replication. The results presented here are the first to demonstrate that *T. thermophila* has the ability to convert hairpin molecules to palindromic molecules during macronuclear development.

DISCUSSION

In this study, we have found evidence that homologous intramolecular recombination is a major pathway by which rDNA palindromes are formed in *T. thermophila*. We constructed heteroduplex rDNA molecules and injected them into developing macronuclei. From such injections, we frequently observed transformants with heterodimeric rDNA, indicating that the two halves of the palindrome are derived from the two complementary strands of the injected rDNA. This result strongly supports the idea that after excision of the rDNA from its micronuclear chromosome, the two strands of the excised rDNA molecule are covalently joined as a giant hairpin molecule by an intramolecular recombination reaction. Although the exact details are not known, we know from previous work that a pair of short inverted repeats located at the 5′ end of the excised molecule are necessary for this reaction (48). In Fig. 2B, we diagrammed two possible ways that inverted repeats can guide intramolecular recombination at a chromosome end. In the first, intrastrand pairing of the inverted repeats forms a cruciform structure. Strand exchange within the cruciform would complete the formation of the hairpin. In the second, a 5′-to-3′ exonuclease forms a 3′ overhang that includes the inverted repeat region. The inverted repeat then folds back on itself, nonhomologous sequences are eliminated, and any gap is filled in by a DNA polymerase. Our results provide the first

clear example of intramolecular, interstrand recombination in the formation of a palindrome.

From microinjection of the heteroduplex rDNA, we also recovered transformants with homodimeric rDNA. Although such palindromes can be formed by a replication-based mechanism, we think that this is unlikely. As put forth in Fig. 2A, the replication model predicts that each transformant will have both types of homodimeric rDNA. However, most transformants generally had only one type. Given that both types of homodimers can be maintained within the same macronucleus and that complete assortment to one type is unlikely in the short time that transformants were grown without subcloning (26), we considered the possibility that homodimers are formed by a mechanism other than replication. Although the SSA mechanism can produce homodimers if the 5′-to-3′ exonuclease reaches the region of the heteroduplex, experiments designed to test this possibility indicated that it is not a likely explanation. A more likely possibility is mismatch repair. If the mismatch of the heteroduplex is repaired before palindrome formation begins, even a recombination-based mechanism would form homodimeric rDNA. We tested this idea by injection of an in vitro-constructed hairpin molecule containing a mismatch and clearly demonstrated that single-base mismatches are repaired in developing macronuclei (see Fig. 6). Repair occurs frequently and can easily account for all of the homodimers observed. The hairpin experiment also demonstrated that *T. thermophila* has the ability to convert a giant hairpin to a giant palindrome—the final step of our model. Thus, a complete picture of palindrome formation is beginning to emerge.

The fate of new ends resulting from chromosome breakage is a useful context for thinking about our results. In *T. thermophila*, micronuclear chromosomes are broken at hundreds of specific sites during development of the macronucleus (see reference 45 for a review). At most of these sites, a new telomere is added to the broken end. The 5′ end of the rDNA is the only known exception. The presence of short inverted repeats near this breakage site efficiently promotes the formation of a hairpin. In rare instances, or if the inverted repeats are somehow impaired, palindromes are not formed, and a new telomere is added at this site instead (48). Thus, one can view telomere formation and hairpin formation as alternative solutions to repairing the broken end next to an inverted repeat, with hairpin formation being the predominant outcome.

In most other eukaryotes, chromosome breakage is an unregulated but common event, resulting primarily from environmental insult or mistakes in normal chromosome metabolism. How cells deal with a broken end is an important issue with regard to cell survival. Work with yeast and *Xenopus* cells suggests that broken ends are highly recombinogenic and that chromosome breaks are usually repaired by recombination with an intact homolog (5, 28). More rarely, broken chromosomes are repaired by the de novo addition of telomeric DNA (4, 16, 19, 23, 32, 43). Broken ends probably also engage in illegitimate recombination, leading to gross chromosome rearrangements like translocations, inversions, and deletions (27). Our results raise the possibility that hairpin formation is another important way that eukaryotic cells process broken ends.

How common might hairpin formation be? Two other clear examples of DNA breakage and hairpin formation are found in the replication cycle of vaccinia virus and the formation of coding ends during V(D)J recombination in lymphoid cells (25, 33). However, the mechanism underlying these reactions is probably different from that in *T. thermophila*, since short inverted repeats are apparently not involved. Hairpin formation by the model that we have proposed might be more widespread than anticipated. Our model requires the presence of short inverted repeats of any sequence next to a chromosome break. This may seem like a rare arrangement in eukaryotic chromosomes. However, a recent sequencing study raises the provocative possibility that short inverted repeats are scattered with surprising frequency throughout eukaryotic chromosomes. In a 2.2-Mb region of *Caenorhabditis elegans* chromosome III, short inverted repeats (average repeat length of 70 bp and spacer length of 164 bp) were found approximately every 5.5 kbp (44). If the foregoing is true for other eukaryotes, then spontaneously formed chromosome breaks would be near short inverted repeats rather often. Given that homologous recombination is a ubiquitous process, hairpin formation through an intramolecular recombination reaction may be a common fate for broken ends.

The notion of hairpin formation at a broken end has implications for the mechanism of gene amplification and, perhaps, for other kinds of genomic rearrangements. One notable feature of amplified genes is that they are frequently found as parts of large palindromes (10). A chromosome fragment that has a hairpin at one end will be easily converted to a palindromic molecule by conventional DNA replication. Palindromes derived from an acentric chromosomal fragment could amplify simply by unequal segregation at mitosis, particularly if a gene carried by the palindrome confers an advantage in high copy number. A hairpin at one end of a centromere-containing chromosome fragment may cause other kinds of problems. Following replication, the resulting palindromic chromosome will be dicentric. Dicentric chromosomes are highly unstable, presumably as a result of the segregation of the centromeres to opposite poles at mitosis and subsequent rupture of the dicentric chromosome (13, 16, 18, 24). In mammalian cells, dicentric palindromic chromosomes may also be part of a pathway culminating in gene amplification. Such aberrant chromosomes have been observed during the early stages of selection for dihydrofolate reductase gene amplification in Chinese hamster ovary cells (22).

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