Cell, Vol. 58, 887–900, September 8, 1989, Copyright © 1989 by Cell Press

A Novel DNA Deletion–Ligation Reaction Catalyzed In Vitro by a Developmentally Controlled Activity from Tetrahymena Cells

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Summary

Developmentally controlled genomic deletion–ligations occur during ciliate macronuclear differentiation. We have identified a novel activity in Tetrahymena cell-free extracts that efficiently catalyzes a specific set of intramolecular DNA deletion–ligation reactions. When synthetic DNA oligonucleotide substrates were used, all the deletion–ligation products resembled those formed in vivo in that they resulted from deletions between pairs of short direct repeats. The reaction is ATP-dependent, salt-sensitive, and strongly influenced by the oligonucleotide substrate sequence. The deletion–ligation activity has an apparent size of 200–500 kd, no nuclease-sensitive component, and is highly enriched in cells developing new macronuclei. The temperature inactivation profile of the activity parallels the temperature lethality profile specific for Tetrahymena cells developing new macronuclei. We suggest that this deletion–ligation activity carries out the genomic deletions in developing macronuclei in vivo.

Introduction

Genomic rearrangement during nuclear differentiation is an intrinsic part of the developmental cycle of ciliated protozoa. During conjugation, the germline micronucleus undergoes meiosis, fertilization, and postzygotic nuclear divisions, giving rise to nuclear division products destined to differentiate into somatic macronuclei. This differentiation involves extensive processing of the entire genome, which in many ciliates has been shown to include large numbers of site-specific deletion–ligation events throughout the genome (Brunk et al., 1982; Yao et al., 1984; Howard and Blackburn, 1985; Allitto and Karrer, 1986; White and Allen, 1986; Ribas-Aparicio et al., 1987; reviewed in Blackburn and Karrer, 1986). In addition, site-specific chromosomal fragmentation has been demonstrated (reviewed in Karrer, 1986, and Blackburn and Karrer, 1986).

Several of these deletion–ligation events have been analyzed previously by comparing sequences of the unrearranged progenitor/precursor micronuclear genome with the corresponding processed macronuclear genomic segments. In every case examined, a given deletion–ligation event involves a simple deletion of a stretch of the micronuclear genome, with joining of the two flanking regions that become contiguous in the macronuclear genome (Klobutcher et al., 1984; Austerberry and Yao, 1987; Herrick et al., 1987; Forney and Blackburn, 1988). No examples of *trans*-joining, i.e., joining of two regions that were on different chromosomes, have been described (reviewed in Karrer, 1986). Deletions both within (Klobutcher et al., 1984) and outside (Callahan et al., 1984) gene coding regions have been found. The biological function of these rearrangements is unknown (reviewed in Karrer, 1986, and Blackburn and Karrer, 1986).

The lengths of the deleted segments, called IESs (internally eliminated sequences), range from 14 bp to over 500 bp in the hypotrichous ciliate Oxytricha nova (Ribas-Aparicio et al., 1987). In Tetrahymena thermophila, IESs are typically many hundreds to thousands of base pairs long (Brunk et al., 1982; Yao et al., 1984; Howard and Blackburn, 1985; Allitto and Karrer, 1986; White et al., 1986). The sequence specificity of the deletion–ligation has been most thoroughly analyzed in two Oxytricha species (Ribas-Aparicio et al., 1987; Herrick et al., 1987). All 14 examples of IESs in these two species were characterized by a pair of direct repeats, from 2 to 6 bp in length, at each end of the deleted segment. The sequences of the direct repeats differed between different IESs. Following deletion–ligation, only one of the direct repeat sequences was retained. All the deleted sequences had short, sometimes imperfect, inverted repeats just inside or overlapping the direct repeats. A similar arrangement has been found for a variably deleted IES in Paramecium tetraurelia (Forney and Blackburn, 1988). In T. thermophila, in the one sequenced example of an IES, a pair of direct repeats flanked the IES, but inverted repeats were not clearly apparent (Austerberry and Yao, 1987). In this example, one direct repeat was retained, but imperfectly. Variations of a few nucleotides in the precise joining point among different macronuclei were also observed for this Tetrahymena IES (Austerberry and Yao, 1987). In addition, deletion–ligation variability at the level of restriction fragment size differences, which suggests alternative use of different junction sequences, has been observed for several Tetrahymena IESs (Austerberry et al., 1984; Howard and Blackburn, 1985; White and Allen, 1986).

Developmentally controlled deletion–ligation events superficially similar to IES removal occur in a variety of other systems, including recombinations in immunoglobulin and T cell receptor genes (Hood et al., 1985), and in rearrangements within the genes controlling nitrogen fixation in the prokaryote Anabaena (reviewed in Haselkorn et al., 1986). The detailed molecular mechanisms for such deletion–ligation reactions have not been elucidated. Certain spontaneous deletions in E. coli (Albertini et al., 1982) and other site-specific recombination events, including transposition (reviewed in Shapiro, 1983) and lambda phage excision (reviewed in Nash, 1981), also involve terminal direct and/or inverted repeats. Ribas-Aparicio and coworkers (1987) have pointed out that the small size of IESs (as few as 14 bp) rules out an excision mechanism involving looping-out of the IES in double-stranded form analogous to lambda phage excision. Instead, they suggested

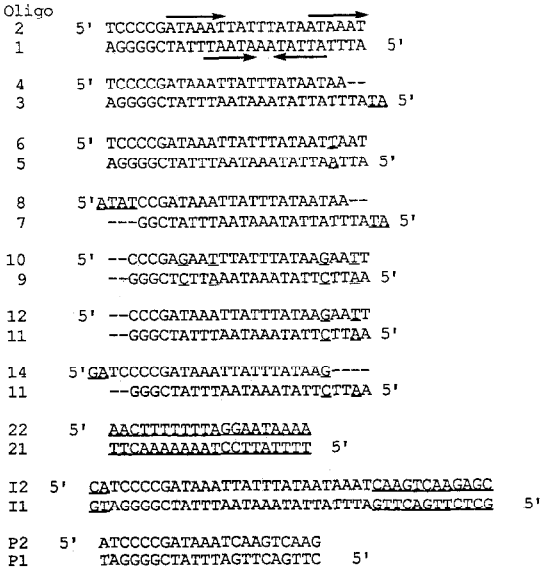


Figure 1. Sequences of Oligonucleotide Substrates Tested

Complementary oligos 1 and 2 consist of the 14 nucleotide IES sequence from O. nova (Ribas-Aparicio et al., 1987), the pair of 6 bp direct repeats that border it (arrows above oligo 2) and 6 bp of the flanking sequence on one side. The inverted repeats associated with this IES are indicated as arrows below oligo 1. Oligos 3–14 are derived from the sequences of oligos 1 and 2: odd-numbered oligos are based on oligo 1, and even-numbered oligos on oligo 2. Their differences relative to the oligo of origin are indicated by underlines, indicating a base change, or by dashes, indicating missing bases. Oligos 21 and 22 are complementary oligos, unrelated in sequence to oligos 1–14 (Spangler et al., 1988). Oligos I1 and I2 are oligos 1 and 2 with additional flanking sequences from the O. nova micronuclear sequence. P1 and P2 are oligos complimentary to each other and to the 26 nucleotide splice product which would result from the excision of the 14 nucleotides in vivo IES from I1 and I2. Differences in their sequences relative to oligos 1 and 2 are not indicated.

that during excision the IES is looped out in single-stranded form.

To determine the mechanism of this unusual type of deletion and ligation, we analyzed the ability of extracts from Tetrahymena thermophila cells to carry out deletion–ligation reactions in vitro, using synthetic DNA oligonucleotide substrates. We report here the finding of a novel activity in Tetrahymena cell-free extracts that carries out a highly specific set of deletion–ligation reactions. These reactions result in removal of the DNA between a pair of direct repeats, with the product retaining one of the repeat sequences. Based on the results reported here, we propose that this novel in vitro activity carries out the in vivo genomic deletion–ligation events that occur during macronuclear development.

Results

Deletion–Ligation Products Formed In Vitro from Synthetic DNA Oligonucleotides in Reactions with Tetrahymena Extracts

As model substrates for in vitro reactions, synthetic DNA oligonucleotides were used to attain high substrate concentrations in the reactions and to allow direct sequencing of products. We initially tested a pair of 26 nucleotide complementary synthetic DNA oligonucleotides. Their sequence consisted of a 14 bp IES, the direct repeats that form its boundaries, and six nucleotides of flanking sequence from the micronuclear genome of Oxytricha nova, as determined by Ribas-Aparicio et al. (1987). The sequences of these model oligonucleotides, called oligos 1 and 2, are shown in Figure 1. We reasoned that, as they contained all of the features noted in common among IESs (Ribas-Aparicio et al., 1987), sufficient *cis*-acting information necessary for IES deletion and ligation of the flanking sequences may be present in these short substrates.

Two procedures were used to prepare extracts from Tetrahymena cells: one based on that used for purification of telomerase from Tetrahymena (Greider and Blackburn, 1987), and one similar to procedures used for in vitro DNA replication in mammalian cell extracts (Li and Kelly, 1984). Both preparations produced similar results, as did extracts further fractionated by column chromatography (see below). Reactions were assayed using oligonucleotide substrates ^{32}P end–labeled at either the 5′ end with polynucleotide kinase or at the 3′ end with a single [α-^{32}P]-dideoxyadenylate residue added by terminal transferase. In a typical reaction either oligo 1 or oligo 2 was 5′ end–labeled with ^{32}P, added to a mixture of excess unlabeled oligos 1 and 2 in equimolar amounts, and the oligonucleotides allowed to anneal. These were added at time zero to a reaction mixture containing an S100 fraction of an extract made from mated Tetrahymena cells that were developing new macronuclei, and an ATP-regenerating system. The products were analyzed by electrophoresis on a 12% polyacrylamide 8 M urea DNA sequencing gel. Figure 2a shows a time course of such a reaction. Although prominent nuclease digestion products were seen migrating faster than the input labeled substrate, longer oligonucleotides were also formed during the reaction. Bands of approximately twice the length of the input oligonucleotides were seen. Sequence analysis of these bands indicated that they consist of a mixture of end-to-end ligated oligos 1 and 2 (data not shown). Strikingly, with 5′ end–labeled oligo 2, three prominent new bands, marked A, B, and C, which migrate between the monomer input oligonucleotide and the dimers, were consistently formed in the reactions (Figure 2a). Measurement of radioactivity in band A versus input oligo showed that 0.3% of the input oligo 2 remaining after a 20 min reaction was converted to product A. Production of A, B, and C was linearly dependent on the amount of the Tetrahymena cell extract; in addition, an ATP-regenerating system consisting of rATP, pyruvate kinase and phosphoenolpyruvate, and both oligonuclotides were required (Figure 2b and data not shown). The production of slow-migrating products is seen in the absence of extract (Figure 2b, lane 2); this is most likely due to impurities in the ATP-regenerating system, as such products were not seen in the absence of the ATP regenerating system (Figure 2, lane 1). With increased time of reaction (Figure 2a), additional bands appeared, marked A-1 and B-1, apparently consisting of A

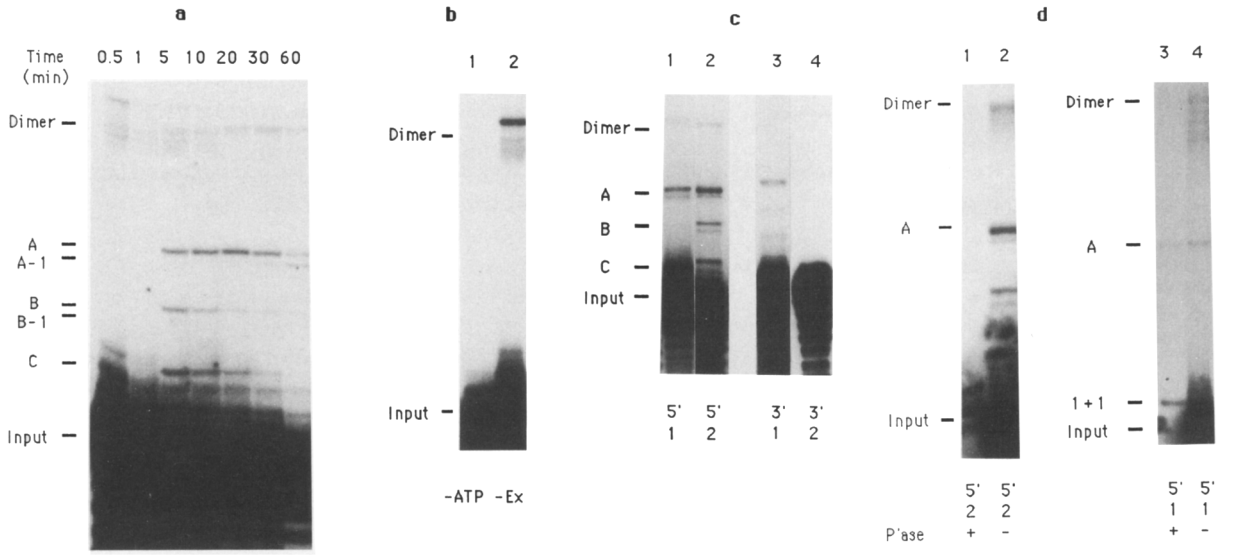


Figure 2. Products of the In Vitro Reaction Catalyzed by Tetrahymena Cell-Free Extracts

(a–d) Equimolar amounts of oligos 1 and 2 were annealed in the presence of tracer amounts of one end-labeled oligo. These were added at a final concentration of 1 μM to a reaction mixture containing cell extracts made from mated T. thermophila, reaction buffer, and an ATP regenerating system and incubated at 30°C as described in Experimental Procedures.

(a) Time course of the reaction. ^{32}P 5′ end–labeled oligo 2 was used as tracer in reactions that were incubated at 30°C for the time indicated. Products were fractionated by electrophoresis on a 12% polyacrylamide 8 M urea DNA sequencing gel and autoradiographed. Bands labeled input, dimer, A, B, C, A-1, and B-1 are described in the text. The products larger than the dimer band were present in control incubations without added Tetrahymena extract (see b).

(b) Requirement for both an ATP-regenerating system and Tetrahymena extract. The 20 min reactions were conducted as above with 5′ end–labeled oligo 2, in the absence of either the ATP-regenerating system (lane 1) or the Tetrahymena mated cell extract (lane 2).

(c) Labeled products resulting from 5′ and 3′ end–labeled oligo substrates. Products of 20 min standard reactions in which the tracer was either 5′ end–labeled oligo 1 or 2 are shown in lanes 1 and 2, respectively. Products resulting from either oligo 1 or 2 3′ end–labeled with ^{32}P dideoxy ATP are shown in lanes 3 and 4, respectively.

(d) Internalization of 5′ end label from oligo 1 substrate. Products from reactions containing either 5′ end–labeled oligo 1 or 2 were treated with calf intestinal phosphatase. Lanes 1 and 2 contain 5′ end–labeled oligo 2 with and without phosphatase treatment, respectively; lanes 3 and 4 contain 5′ end–labeled oligo 1 with and without phosphatase treatment, respectively.

and B each shortened by one nucleotide. This was confirmed directly by DNA sequencing (data not shown).

Lanes 1 and 2 of Figure 2c show the reaction products of typical reactions, in which either oligonucleotide 1 or 2 was 5′ end–labeled, preannealed, and reacted as described above. Whereas the three products A, B, and C were observed with 5′ end–labeled oligo 2 (lane 2), with 5′ end–labeled oligo 1 only a single band comigrating with band A (and some A-1 band) was seen in addition to the dimer bands, input oligonucleotide, and nuclease breakdown products (lane 1).

The production of oligonucleotides longer than the input substrate oligonucleotides was unexpected, and suggested involvement of a ligation step. Therefore, we treated the reaction products with calf intestinal phosphatase. When the 5′ end–labeled substrate was oligo 2, the label in bands A, B, and C was completely removed by phosphatase treatment (Figure 2d, lane 1). In contrast, when oligo 1 was the 5′ end–labeled substrate, the label in band A was resistant to such treatment (lane 3). This indicated that the 5′ end-label of oligo 1 was in an internal position in the polynucleotide chain labeled band A. In addition, internalized ^{32}P label was also found in a novel band approximately one nucleotide longer than input oligo 1 (marked 1+1 in lane 3). Although some weak bands resistant to phosphatase were seen below the input oligo 1 band (data not shown), nuclease breakdown products smaller than the input oligos made it difficult to analyze such small quantities of products in this size range.

Lanes 3 and 4 of Figure 2c show the products observed when the labeled substrate oligos were 3′ end–labeled. In lane 3, three bands, corresponding to but slightly longer than the bands A, B, and C in lane 2, were seen with 3′ dideoxy A–labeled oligo 1 substrate. However, no labeled reaction products longer than the input oligo were seen when the input was 3′ dideoxy A–labeled oligo 2 (lane 4).

The sequences of bands A, B, and C were determined by the method of Maxam and Gilbert (1980) from the products of input 5′ end–labeled oligo 2. Each of these three product bands, called products A, B, and C, was a unique species. Their sequences are shown in Figure 3a. The sequence of product A was confirmed using 3′ end–labeled oligo 1. When product A from 5′ end–labeled oligo 1 was subjected to Maxam–Gilbert sequencing, unreadable sequencing gels resulted, confirming the expectation that this product has an internal phosphate label.

The sequence analysis, together with the retention of the 5′ and 3′ ^{32}P labels of input oligo 1 in product A,

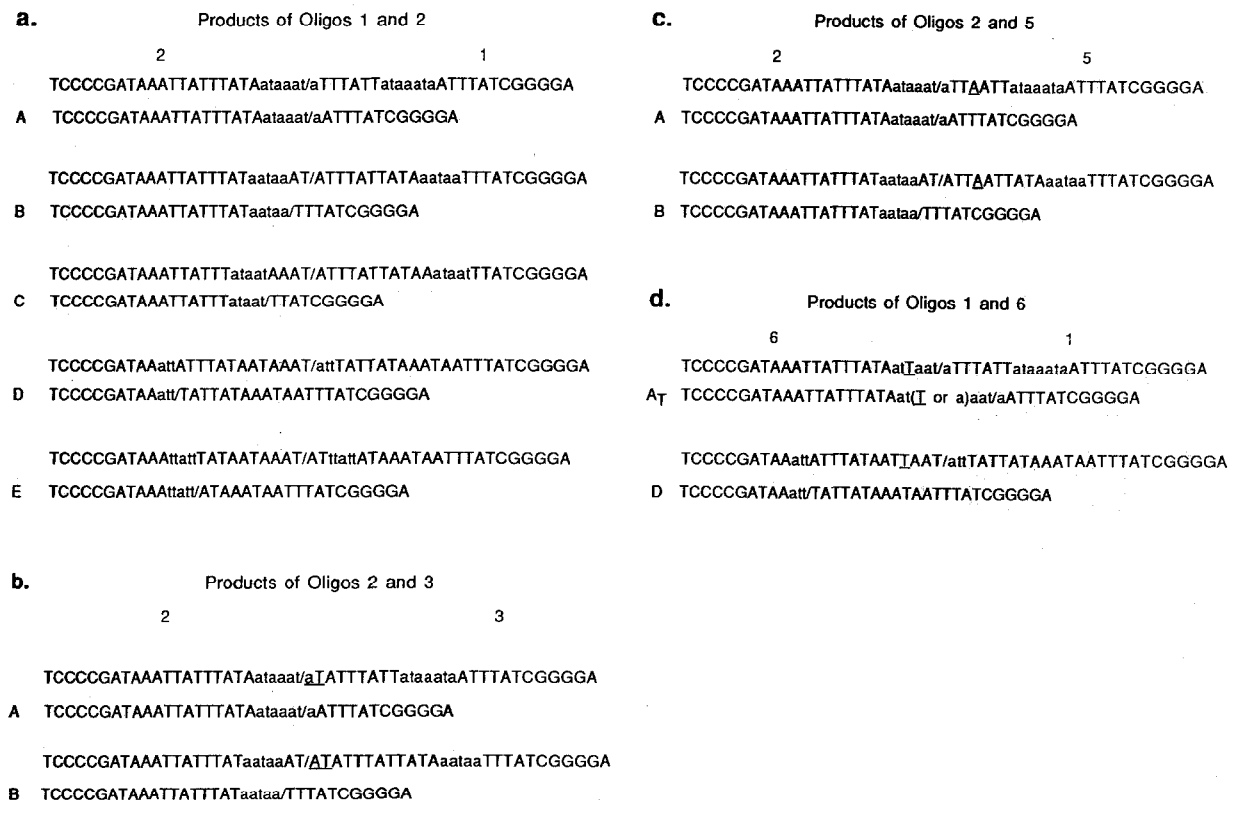


Figure 3. Product Sequences

The figure lists the sequences of deletion–ligation products from various combinations of DNA oligo substrates, as determined by Maxam–Gilbert sequencing. The sequence of the relevant putative dimer is shown above each product. Direct repeats are in lowercase. Sequence differences compared with oligo 1 or 2 are underlined. (a) Products of oligos 1 and 2. (b) Products of oligos 2 and 3. (c) Products of oligos 2 and 5. (d) Products of oligos 1 and 6.

demonstrated that the 39 nucleotide product A consists of the entire sequence of oligo 2 joined at the 3′ end either to the 5′ A residue plus the 12 nucleotides at the 3′ end of oligo 1, or to the 13 nucleotides at the 3′ end of oligo 1. Product B (35 nucleotides) consists of the sequence of oligo 2, minus the 2 nucleotides from its 3′ end, joined at the 3′ end to the 11 nucleotides from the 3′ end of oligo 1. Product C (32 nucleotides) consists of the sequence of oligo 2, minus the four nucleotides from its 3′ end, joined at the 3′ end to the 10 nucleotides from the 3′ end of oligo 1. The appearance of label in products A, B, and C when the labeled substrate oligo was 3′ end–labeled oligo 1, or 5′ end–labeled oligo 2, is consistent with each of these products containing both the 3′ region of oligo 1 and the 5′ end of oligo 2.

Taken together, these data suggest that products A, B, and C result from deletions within a dimer of oligos 2 and 1 ligated end-to-end in the orientation shown in Figure 3a. Strikingly, the deletions that yield products A, B, and C all take place between pairs of direct repeats that result from dimer formation. One direct repeat sequence is retained in each of the products, and the other is deleted along with the intervening DNA. Each product apparently utilizes a unique direct repeat; these are indicated by lowercase letters in Figure 3a, and are 7, 5, and 5 nucleotides in length for products A, B, and C, respectively.

We attempted to detect deletion of the 14 nucleotide IES that is deleted in O. nova using Southern analysis of products formed in the in vitro reactions. For these experiments, the substrates were synthetic DNA oligonucleotides longer than oligos 1 and 2, as we reasoned that additional sequences that flank this IES in the O. nova genome may be necessary for correct deletion. These complementary 40 nucleotide oligos, I1 and I2 (Figure 1), include all of the sequences of oligos 1 and 2, respectively, plus additional Oxytricha micronuclear sequences on both sides of oligos 1 and 2. Equimolar amounts of unlabeled oligos I1 and I2 were preannealed and carried through the same reaction as described above for oligos 1 and 2 (again, final oligo concentrations were 1 μM). The products were separated by electrophoresis on a 12% denaturing polyacrylamide sequencing gel, electroblotted, and duplicate filters probed separately with 5′ end–labeled 22 nucleotide oligos (P1 and P2, see Figure 1) complementary to either strand of the expected 26 nucleotide oligo that would result from deletion of the 14 nucleotide O. nova IES from the input oligos. In each case, the complementary 22 nucleotide oligo was run on the same gel

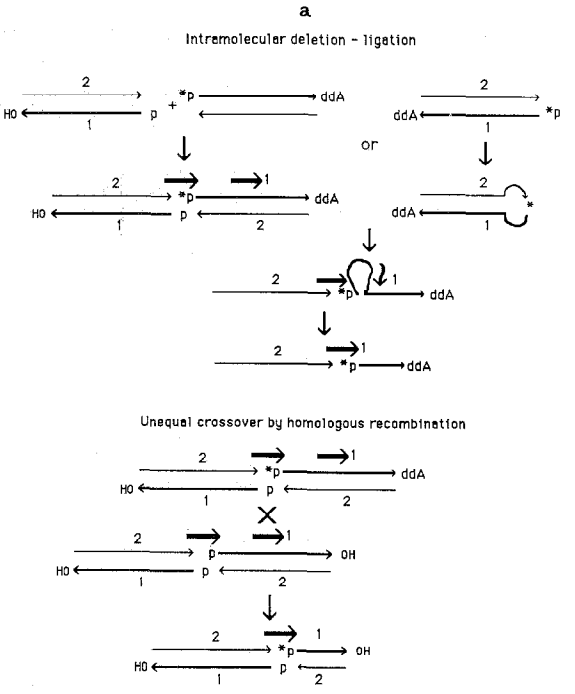


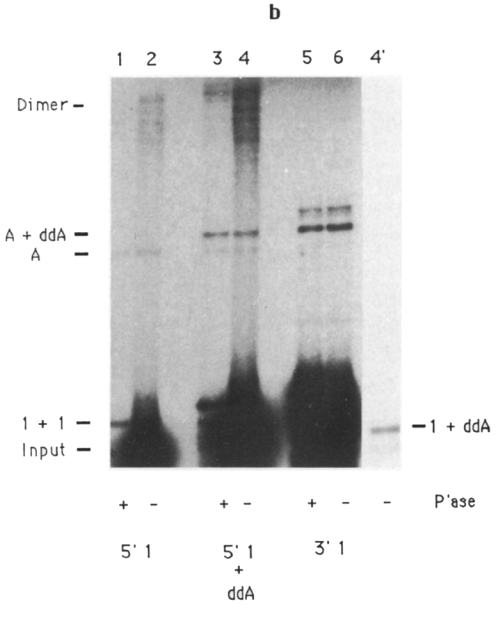
Figure 4. Distinction between Intra- and Intermolecular Deletion–Ligation Mechanisms

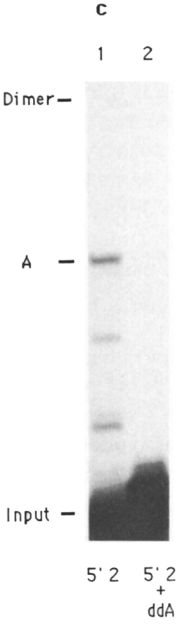
(a) Schematic representation of the test experiment used to distinguish between a reaction involving an intramolecular deletion step and an intermolecular reaction involving unequal crossover via homologous recombination. Oligos 1 and 2 are shown as heavy or light arrows, respectively, with the 5′ to 3′ orientation indicated by the direction of the arrow. * P, 5′ ^{32}P label on oligo 1; ddA, dideoxyadenylate residue added to "tag" the 3′ end of oligo 1; short heavy arrows, the 7 nucleotide direct repeats that may be involved in product A formation (see text and Figure 3a).

Top: Formation of oligo 2–1 dimer and intramolecular deletion within oligo 1. Oligos 2 and 1 are joined end-to-end, either by blunt-end ligation of two duplex molecules (left), or by ligation of one end of a single oligo 2 and 1 duplex (right). Intramolecular deletion of the 13 nucleotide sequence between the 5′ ^{32}P and the 3′ dideoxy A tag, followed by ligation of the flanking nucleotides on the same oligo molecule, forms product A molecules carrying both the ^{32}P label and the dideoxy A tag. Bottom: In contrast, dimerization of two oligo 2–1 duplexes by blunt end ligation followed by homologous but unequal crossover at the direct repeats on two such molecules separates the ^{32}P label from the dideoxy A tag in product A molecules. This is predicted because unlabeled, untagged oligo 1 substrate molecules are added in excess. Thus, individual product A molecules would contain either the ^{32}P label or the dideoxy A tag, but not both.

(b) Results of the test diagrammed in (a). Lanes 1 and 2, control reactions using untagged, ^{32}P 5′ end–labeled oligo 1 as the tracer, with (lane 1) or without (lane 2) calf intestinal phosphatase treatment; lanes 3 and 4, test reaction using tracer oligo 1 that was both dideoxy A 3′ end-tagged and ^{32}P 5′ end–labeled, with (lane 3) or without (lane 4) calf intestinal phosphatase treatment; lanes 5 and 6, control reaction using tracer oligo 1 that was ^{32}P labeled at its 3′ end with ^{32}P dideoxy A, but carried a 5′ OH, with (lane 5) or without (lane 6) calf intestinal phosphatase treatment. The structure of the labeled band above the A + ddA band in lanes 5 and 6 is unknown; in some experiments it was not detectable. Lane 4′, short exposure of lane 4, to show position and relative amounts of input 5′ end–labeled oligo 1 with and without the 3′ dideoxy A tag.

(c) Effect on product A, B, and C formation of blocking the 3′ end of ^{32}P 5′ end–labeled oligo 2 with a dideoxy A residue. 5′ end–labeled oligo 2 without (lane 1) or with (lane 2) a 3′ dideoxy A residue in reactions carried out as usual.





as a positive control. However, no 26 nucleotide product complementary to either P1 or P2 was detected, even though the control reconstructions were detectable at levels as low as 0.2% of the concentration of the input oligo remaining after the reaction (data not shown).

Formation of Product A by an Intramolecular Reaction

By analogy with the mechanism suggested by Ribas-Aparicio et al. (1987), the deletions that form products A, B, and C could result from an intramolecular looping-out of the deleted region between the direct repeats in the putative dimer formed by end-to-end joining of oligos 2 and 1. We wished to distinguish such a mechanism from another possibility, homologous recombination by unequal crossover between one of the direct repeats on a duplex oligo 2–1 dimer molecule with the other repeat on another identical molecule, which would also result in removal of the sequence between the repeats and retention of one repeat in the product. This latter mechanism would be intermolecular. These two possible mechanisms are schematically illustrated in Figure 4a. To determine whether the reaction was intramolecular or intermolecular, a double end-labeling experiment was designed.

Because of the high concentration of rATP in the reaction (4 mM), it is highly unlikely that ^{32}P label is transferred from one DNA molecule to another (via phosphatase and kinase activities in the crude S100 fraction) without a very large dilution with unlabeled phosphate. Consistent with this, in products A, B, and C the ^{32}P 5′ end label in oligo 2 was found only at the 5′ ends of the products. Therefore, we conclude that, when 5′ end–labeled oligo 1 is the substrate, the internal ^{32}P label in product A is that originally at the A residue at the 5′ end of oligo 1, and does not result from exchange between molecules.

As described above, product A is labeled by the ^{32}P from the 5′ end of substrate oligo 1, or by the ^{32}P-labeled dideoxy A residue added to the 3′ end of oligo 1. Together with the sequence analysis, these data show that product A contains the ^{32}P phosphate group, and possibly the 5′ A residue as well, from the 5′ end of oligo 1, as well as the 12 or 13 nucleotides from its 3′ end (Figure 3a). Hence, if product A is the result of an intramolecular reaction, it should include both the 3′ and 5′ ends of a single oligo 1 molecule.

In the double end–labeling experiment shown schematically in Figure 4a, oligo 1 was ^{32}P labeled to high specific activity at its 5′ end, then the 3′ ends of these 5′ end–labeled molecules were "tagged" with a single unlabeled dideoxy A residue. These labeled and tagged oligo 1 molecules were purified away from components of the labeling reactions, then diluted ~40 fold with unlabeled oligo 1. An equimolar amount of unlabeled oligo 2 was added and the oligos were annealed as usual prior to reaction with the extract. The dilution ensured that the probability of reaction of a tagged and labeled oligo 1 molecule with another tagged and labeled molecule was very low. Therefore, we predicted that if an intermolecular reaction occurred, the 5′ end label in product A would be separated from the 3′ dideoxy A tag. In contrast, an intramolecular reaction, in which a 13 nucleotide internal portion of oligo 1 is removed, would produce product A molecules containing both the 5′ ^{32}P label and the 3′ dideoxy A tag.

Figure 4b shows the result of this experiment. The reaction produced product A molecules that contained both the ^{32}P label and the 3′ dideoxy A tag of the input substrate oligo 1. This was evident because product A carrying the extra dideoxy A residue at its 3′ end (marked A+ddA in Figure 4, lanes 3–6) is longer by one nucleotide than a marker product A without it (lanes 1 and 2). The labeled dideoxy A–tagged product A in the test experiment (lanes 3 and 4) comigrated with marker product A from input oligo 1 substrate that was 3′ end–labeled with ^{32}P dideoxy A (lanes 5 and 6). Confirmation that the reaction was intramolecular came from densitometric analysis of the ratio of input 5′ end–labeled oligo 1 with and without the 3′ dideoxy A tag, using a shorter exposure of the autoradiogram (Figure 4b, lane 4′). This ratio of 2.15:1 was not significantly different from the measured ratio (2.14:1) of ^{32}P labeled product A molecules with and without the 3′ dideoxy A tag (bands marked A+ddA and A in lane 4). This is the result expected if there has been no dilution of ^{32}P label in product A with unlabeled untagged oligo 1. Therefore we conclude that, in the reaction resulting in product A (and very likely products B and C as well, see below), the 5′ end of oligo 1 is ligated to the 3′ end of oligo 2. This is followed, in the simplest pathway (shown in Figure 4a), by a deletion of 13 internal nucleotides, including one of the 7 nucleotide direct repeats and ligation of the flanking nucleotides to form product A.

The above experiments did not directly demonstrate whether ligation of oligo 2 to oligo 1 is required to form B and C. The lack of label in products A, B, and C when 3′ dideoxy A–labeled oligo 2 was used (Figure 2c, lane 4) is consistent with the hypothesis that blocking the 3′ end of this oligo by the labeled dideoxy A residue prevents formation of an oligo 2-oligo 1 dimer intermediate, but does not exclude the possibility that the terminal dideoxy A residue is removed during product formation. This was tested directly by using ^{32}P 5′ end–labeled oligo 2 blocked with a single unlabeled dideoxy A residue at its 3′ end. No labeled products A, B, or C were formed from this substrate (Figure 4c, lane 2), in contrast to the control reaction with 5′ end–labeled, unblocked oligo 2 (lane 1). Together, these results indicate that end-to-end joining of oligos 2 and 1 is necessary to form products A, B, and C.

Developmental Control and Enzymic Properties of the Deletion–Ligation Activity

The reaction producing products A, B, and C had two unexpected properties: the apparent requirement for a dimer intermediate and no detectable deletion of the 14 nucleotide IES of Oxytricha nova. To determine whether the in vitro activity producing A, B, and C had other properties expected for the activity that removes IESs in vivo, we used two approaches to analyze the reaction further: characterization of the deletion–ligation activity and examination of the substrate requirements.

We first investigated the developmental control of the

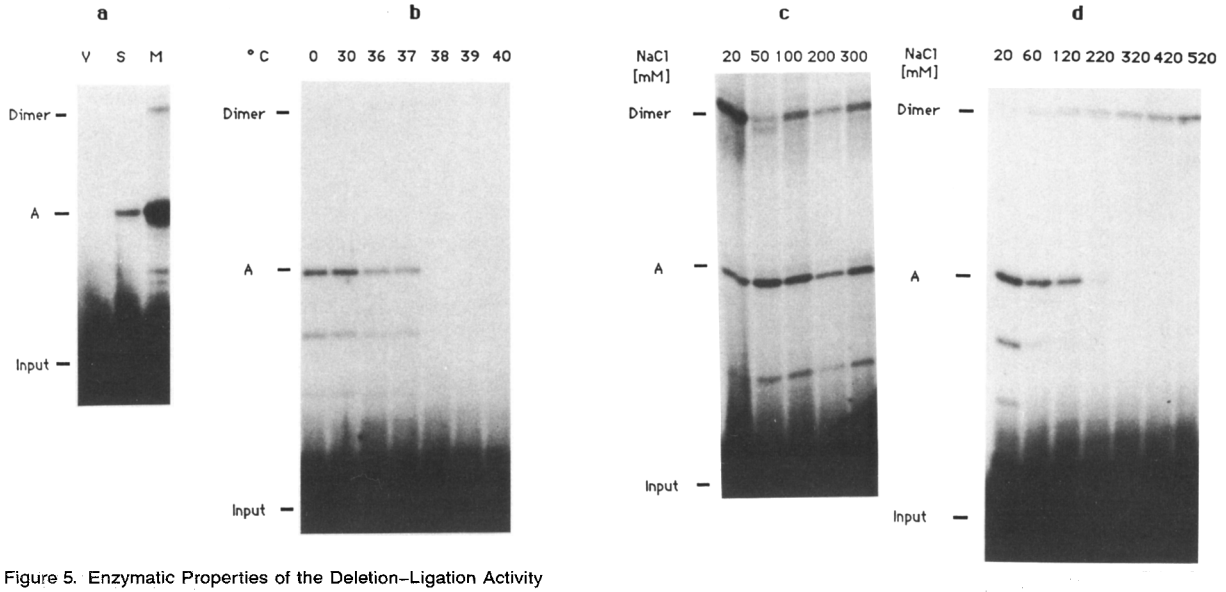


Figure 5. Enzymatic Properties of the Deletion–Ligation Activity

(a) Developmental control. Assays were performed using an S100 fraction of extracts prepared as described in Experimental Procedures from vegetatively growing cells (V), cells which had been starved but not mated (S), and mated cells (M). 5′ end–labeled oligo 2 was the tracer.

(b) Temperature sensitivity. Extracts were preincubated for 20 min at the indicated temperatures. Oligos were then added to initiate the reaction, which was carried out at 30°C. The tracer was 5′ end–labeled oligo 2.

(c) Sensitivity to prior exposure to salt. Extracts were preincubated in the indicated NaCl concentrations at 0°C for 20 min. Samples were then brought to 60 mM in NaCl and assayed as usual. The tracer was 5′ end–labeled oligo 2.

(d) Salt sensitivity of the deletion–ligation reaction. Reactions were conducted as usual in the presence of the indicated concentrations of NaCl. The tracer was 5′ end–labeled oligo 2.

specific deletion–ligation activity. Figure 5a shows the results of assays performed on S100 fractions of extracts made in parallel from mated cells (lane M), cells that had been starved but not mated (lane S), and exponentially growing vegetative cells (lane V). Starvation is required to induce mating in Tetrahymena. The levels of deletion–ligation activity were reproducibly much higher in mated compared with nonmated cells, and there was little activity in the extract isolated from vegetatively growing cells. The mated cell extracts were made 12 hr after mating was initiated, at which time macronuclear genomic rearrangements, including IES deletions, are in progress (Austerberry et al., 1984; Allis et al., 1987). Hence, the activity responsible for these deletions is expected to be present during this same time period.

Preliminary experiments suggested that the temperature optimum of the specific deletion–ligation reaction in vitro was about 30°C (data not shown), the optimal temperature for Tetrahymena growth and mating. The thermal stability of the activity was tested by preincubating extracts at different temperatures, adding the oligo substrates, and then assaying them at 30°C. Activity was present in extracts that had been preincubated at temperatures up to and including 37°C, but was completely destroyed by preincubation at 38°C or higher (Figure 5b). We note that in genetic experiments, Scholnick and Bruns (1982) demonstrated that only Tetrahymena cells in the process of developing new macronuclei are specifically and quantitatively killed by exposure to temperatures at or above 38°C, in contrast with vegetative cells, which are completely viable after comparable treatment.

Under our standard conditions, the final NaCl concentration in the assay reaction was 40 mM. To determine whether the activity was inactivated by prior exposure to high salt, we preincubated extracts in various concentrations of NaCl before assaying in 60 mM NaCl. As is evident in Figure 5c, prior exposure to NaCl concentrations as high as 300 mM had no effect on the deletion–ligation activity.

To determine the effect of NaCl concentration on the reaction, we assayed the deletion–ligation activity in the presence of NaCl concentrations ranging from 20 mM to 520 mM. Similar amounts of deletion–ligation products were formed in the presence of 20 and 60 mM NaCl, somewhat less in 120 mM NaCl, very little in 220 mM NaCl, and none under higher NaCl concentrations (Figure 5d and data not shown).

Fractionation and Nuclease Insensitivity of the Deletion–Ligation Activity

We initiated purification of the deletion–ligation activity by gel filtration chromatography of the S100 fraction of Tetrahymena extracts on a Bio-Rad Bio-Gel A-5m sizing column. These extracts were made in TMG buffer containing 0.1 M NaCl. The peak of activity occurred at 200,000–500,000 daltons (Figure 6a). When an extract made in TMG buffer without 0.1 M NaCl was run on the column, no activity was initially detectable; however, very long autoradiographic exposures showed very low levels of activity spread throughout all fractions (data not shown). The possibility that these results were due to a suboptimal NaCl concentration during the assay was tested by adding

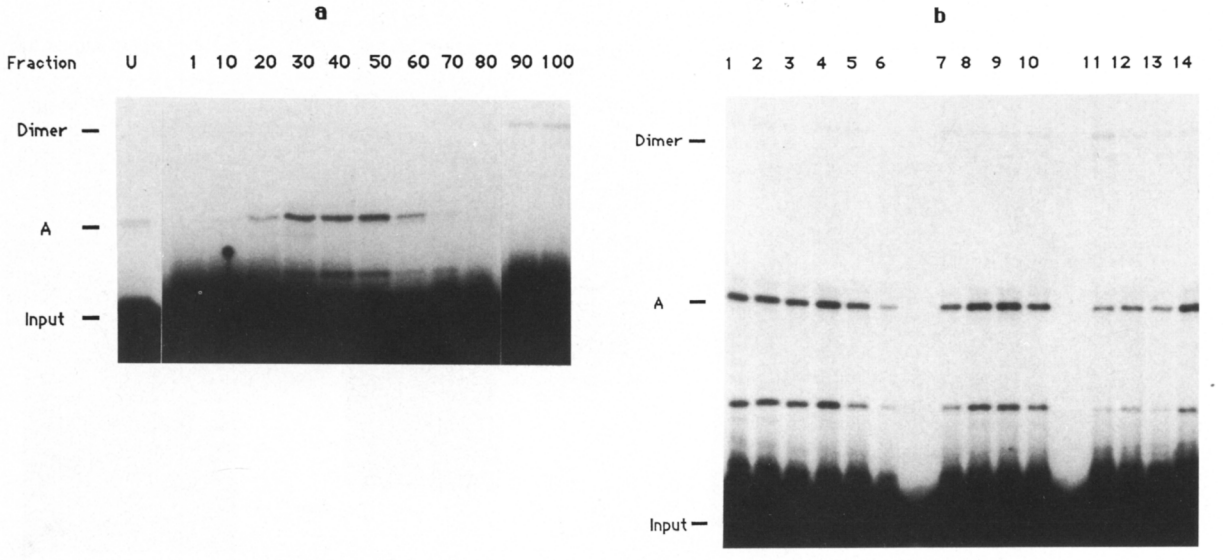


Figure 6. High Molecular Weight and Nuclease Insensitivity of the Deletion–Ligation Activity

(a) Fractionation on a sizing column. Mated cell extracts were run on a 78 cm Bio-Gel A-5m sizing column that had been precalibrated with Bio-Rad protein molecular weight standards (Greider and Blackburn, 1987). The 670 kd standard eluted in fraction 23, 95 kd in fraction 57, and 17 kd in fraction 75. Fractions were collected and assayed as usual, using 5′ end–labeled oligo 2 as tracer. U, unfractionated extract.

(b) Nuclease insensitivity of the activity. An active Bio-Gel A-5m column fraction was preincubated at 30°C for 20 min in the presence of various concentrations of RNAase A or micrococcal nuclease. Following inactivation of the nucleases, assays were conducted as usual, using 5′ end–labeled oligo 2 as tracer. Lanes 1–6: preincubation mixtures were treated with RNAase A at final concentrations of 2 mg/ml (lanes 1 and 4), 200 ng/ml (lanes 2 and 5), and 20 ng/ml (lanes 3 and 6). Lanes 1–3 were treated with 3 U/λ RNasin after preincubation; lanes 4–6 were treated with 3 U/λ RNasin prior to preincubation. Lanes 7–10 were treated with oxidized RNAase A: lanes 7 and 9 with 2 μg/ml, and lanes 8 and 10 with 200 ng/ml. Lanes 7 and 8 were treated with RNasin after preincubation; lanes 9 and 10 were treated prior to preincubation. Lanes 11–14 were treated with micrococcal nuclease; lanes 11 and 13 with 0.7 U/λ, and lanes 12 and 14 with 0.07 U/λ. Lanes 11 and 12 were made 4.76 mM in EGTA after preincubation, and lanes 13 and 14 were made 4.76 mM in EGTA before preincubation.

NaCl prior to assaying the fractions that had been purified in the absence of 0.1 M NaCl; again, no significant activity or fractionation were seen (data not shown). Some proteins can be subject to aggregation in low ionic strengths. These results suggest that the deletion–ligation activity resides in or aggregated with such proteins, and thus, in the absence of NaCl, did not fractionate on the sizing column but rather eluted across the entire size profile by slowly leaching into the eluate.

To test whether the deletion–ligation activity has a required RNA or DNA component, active S100 or Bio-Gel A-5m column fractions were preincubated with various concentrations of RNAase or micrococcal nuclease. The nucleases were inactivated prior to assay with either RNasin for the RNAase A experiments, or EGTA for the micrococcal nuclease experiments. Neither RNAase A nor micrococcal nuclease treatment had any discernable effect on the deletion–ligation activity (Figure 6b and data not shown), which indicates that there is no required RNA or DNA component in the activity.

Effect of Relative Concentrations of Oligonucleotide Substrates on Deletion–Ligation Products

Under standard reaction conditions, unlabeled oligos 1 and 2 were present in equal concentrations (1 μM), with one radiolabeled oligo present in tracer amounts. Although the relative amounts of products B and C varied in different experiments and with the particular extract used, under these conditions product A was the major deletion–ligation product formed. When 5′ end–labeled oligo 1 and a 5:1 or 25:1 molar ratio of cold substrate oligos 2 and 1 were used, similar results were obtained (Figure 7a, lanes 3, 8, and 9). However, when we used 5′ end–labeled oligo 2, and a 5:1 molar ratio of cold substrate oligos 1 and 2, two prominent new products (marked D and E) longer that the input oligos were generated, and product A became a minor product (Figure 7a, lanes 6 and 12). In this experiment, absolute as well as relative concentrations of oligos affected the formation of these new products. Lanes 6 and 11 in Figure 7a contain the products of reactions in which the cold oligos were present in a 5:1 ratio of oligo 1 to oligo 2, yet products D and E are not present in lane 11, which has 5-fold less total cold oligo compared with lane 6 (270 ng vs. 1350 ng). It is not until lane 12, in which the ratio of cold 1 to cold 2 is 25:1, but in which the total concentration of cold oligo 1 plus 2 (1170 ng) approaches that of lane 6, that products D and E become prominent.

The ^{32}P label in D and E, derived from 5′ end–labeled oligo 2, was phosphatase sensitive (data not shown). The sequences of D and E (Figure 3a) were determined by the Maxam–Gilbert procedure. Product D (36 nucleotides) consists of the 13 nucleotide sequence from the 5′ end of oligo 2 joined at the 3′ end to the 23 nucleotide sequence

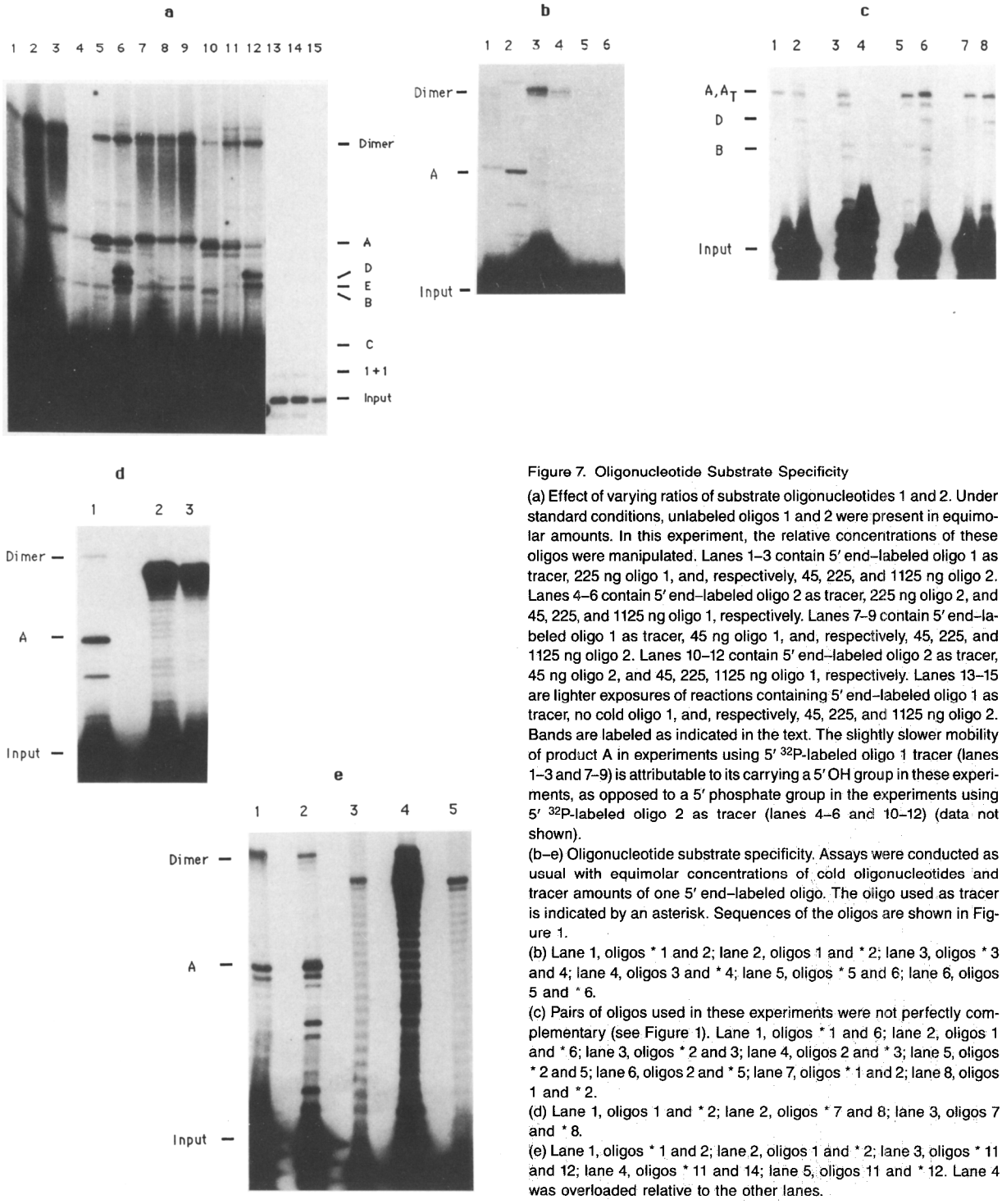


Figure 7. Oligonucleotide Substrate Specificity

(a) Effect of varying ratios of substrate oligonucleotides 1 and 2. Under standard conditions, unlabeled oligos 1 and 2 were present in equimolar amounts. In this experiment, the relative concentrations of these oligos were manipulated. Lanes 1–3 contain 5′ end–labeled oligo 1 as tracer, 225 ng oligo 1, and, respectively, 45, 225, and 1125 ng oligo 2. Lanes 4–6 contain 5′ end–labeled oligo 2 as tracer, 225 ng oligo 2, and 45, 225, and 1125 ng oligo 1, respectively. Lanes 7–9 contain 5′ end–labeled oligo 1 as tracer, 45 ng oligo 1, and, respectively, 45, 225, and 1125 ng oligo 2. Lanes 10–12 contain 5′ end–labeled oligo 2 as tracer, 45 ng oligo 2, and 45, 225, 1125 ng oligo 1, respectively. Lanes 13–15 are lighter exposures of reactions containing 5′ end–labeled oligo 1 as tracer, no cold oligo 1, and, respectively, 45, 225, and 1125 ng oligo 2. Bands are labeled as indicated in the text. The slightly slower mobility of product A in experiments using 5′ ^{32}P-labeled oligo 1 tracer (lanes 1–3 and 7–9) is attributable to its carrying a 5′ OH group in these experiments, as opposed to a 5′ phosphate group in the experiments using 5′ ^{32}P-labeled oligo 2 as tracer (lanes 4–6 and 10–12) (data not shown).

(b–e) Oligonucleotide substrate specificity. Assays were conducted as usual with equimolar concentrations of cold oligonucleotides and tracer amounts of one 5′ end–labeled oligo. The oligo used as tracer is indicated by an asterisk. Sequences of the oligos are shown in Figure 1.

(b) Lane 1, oligos * 1 and 2; lane 2, oligos 1 and * 2; lane 3, oligos * 3 and 4; lane 4, oligos 3 and * 4; lane 5, oligos * 5 and 6; lane 6, oligos 5 and * 6.

(c) Pairs of oligos used in these experiments were not perfectly complementary (see Figure 1). Lane 1, oligos * 1 and 6; lane 2, oligos 1 and * 6; lane 3, oligos * 2 and 3; lane 4, oligos 2 and * 3; lane 5, oligos * 2 and 5; lane 6, oligos 2 and * 5; lane 7, oligos * 1 and 2; lane 8, oligos 1 and * 2.

(d) Lane 1, oligos 1 and * 2; lane 2, oligos * 7 and 8; lane 3, oligos 7 and * 8.

(e) Lane 1, oligos * 1 and 2; lane 2, oligos 1 and * 2; lane 3, oligos * 11 and 12; lane 4, oligos * 11 and 14; lane 5, oligos 11 and * 12. Lane 4 was overloaded relative to the other lanes.

from the 3′ end of oligo 1, while product E (35 nucleotides) contains the 16 nucleotides from the 5′ end of oligo 2 joined at the 3′ end to the 19 nucleotides from the 3′ end of oligo 1. We noted that product E is the complement of product B (see Figure 3a). As with A, B, and C, the deletion–ligation events resulting in D and E occurred between a pair of direct repeats in the putative oligo 2–1 dimer, with only one repeat retained in the product (Figure 3a). The direct repeats were 3 and 5 nucleotides in length for products D and E, respectively. Variable secondary structures within the oligo substrates may explain this alteration in product formation. That secondary structure plays a role in product formation is suggested by the presence of inverted as well as direct repeats in most of the

IESs analyzed thus far (Ribas-Aparicio et al., 1987; Forney and Blackburn, 1988). There is a high degree of self-complementarity in the AT-rich sequences of oligos 1 and 2. By increasing the amounts of oligo 1 relative to oligo 2, interactions between complementary regions on two oligo 1 molecules may become more favorable. Such interactions could lead to a shift in secondary structure in the oligos, favoring the production of products D and E.

When the reaction mixture contained cold oligo 2 and only a tracer amount of oligo 1 ^{32}P-labeled at its 5′ end (i.e., no added cold oligo 1), a product with a phosphatase-insensitive label, comigrating with 26–27 nucleotide oligos, was efficiently produced (marked 1+1 Figure 7a, lanes 13–15). We estimate that the intensity of this band was 5%–10% that of the input oligo 1 band. This band comigrated with the 1+1 band seen in Figure 2d, lane 1. However, no corresponding product was detected in the counterpart experiments using 5′ end–labeled oligo 2 (data not shown). We therefore believe that this product is most likely a ligation intermediate (reviewed in Kornberg, 1980) formed in the pathway producing the oligo 2–1 dimer, in which an AMP is joined by a 5′–5′ pyrophosphate bond to the 5′ end of oligo 1.

Substrate Specificity of the Deletion–Ligation Reaction

That an oligo 2–1 dimer is a reaction intermediate was indicated both by the inhibition of product formation in the presence of a 3′ dideoxy A on oligo 2 and by the sequences of the products. Therefore we tested whether product formation could be stimulated by using as substrates a pair of complementary oligos, oligos 3 and 4 (Figure 1), which form a duplex with complementary overhanging ends. Two such duplexes would be more readily ligated to form correctly oriented dimers than blunt ended duplexes of oligos 2 and 1. The sequences of oligos 3 and 4 were chosen so that the dimer resulting from sticky-ended ligation would have the same sequence as the putative oligo 2–1 intermediate shown in Figure 3. (Use of a preformed 52 nucleotide oligo substrate consisting of the putative oligo 2–1 dimer was not practical in these assays; nuclease breakdown products from such an input dimer would run in the same region as deletion–ligation products.) When oligos 3 and 4 were substituted for oligos 1 and 2 in the reactions, formation of the dimer band was greatly enhanced, as expected. However, no products A–E were detected (Figure 7b, lanes 3 and 4). We therefore conclude that simply ligating a duplex of oligos 3 and 4 to form a molecule with the same sequence as the duplex oligo 2–1 dimer does not alleviate a rate-limiting step in the specific deletion–ligation reaction. We suggest that if dimer formation occurs within a single duplex of complementary oligos rather than by the ligation of two duplexes into a duplex dimer (see Figure 4a), then oligos 3 and 4 might not be as favorable as substrates as oligos 1 and 2, and that the four-base overhang may interfere in some way with efficient product formation by this mechanism. Further work is necessary to determine how dimer formation occurs, and whether a single- or double-stranded form of the dimer is a reaction intermediate. The oligonucleotide substrates were routinely preannealed prior to the deletion–ligation reaction. However, it should be noted here that, in reactions carried out in parallel, oligos that were not preannealed also produced deletion–ligation products, with only an approximate 2-fold reduction in efficiency compared with those which were preannealed (data not shown).

Since each deletion–ligation event occurred between two direct repeats, we examined the effect of alterations in these sequences on the formation of deletion–ligation products. We tested a pair of synthetic complementary oligonucleotides, oligos 5 and 6; their sequences, shown in Figure 1, are identical to those of oligos 1 and 2, except for a single base change in the sequence of oligo 1 at nucleotide 4 to give oligo 5 and the complementary change in oligo 2 at nucleotide 23 to give oligo 6. This change disrupts the direct repeats on the 5′ side of the sequences eliminated from products A, B, and E without affecting those involved in the production of C or D (see Figure 3a). The oligos were 5′end–labeled and carried through the deletion–ligation reaction as described above. The extract was unable to utilize oligos 5 and 6 to form any of products A–E efficiently (Figure 7b, lanes 5 and 6); at most only minor amounts of variable product bands were seen (data not shown).

To determine the effect of a single base change in the putative dimer sequence, we tested oligo 6 plus 1 together in the same reaction, and oligo 2 plus 5. Both combinations resulted in efficient formation of deletion–ligation products (Figure 7c). The major products resulting from 5′ end–labeled input oligos 2 or 6 (Figures 3 and 7c, lanes 2, 5, and 8) were sequenced. These products from 5′ end–labeled 1 or 5 had an internal ^{32}P label, as demonstrated by phosphatase insensitivity, which resulted in unreadable sequencing gels. Oligos 6 plus 1 gave product D, as well as a band comigrating with product A (Figure 7c, lanes 1 and 2). Sequence analysis of this latter band indicated that it did, indeed, consist of product A; however, in the AG sequencing reaction, the intensity of the band in the sequencing gel at the nucleotide 23 position was approximately half that of the other A residue bands in this sequence. Since this was the site at which the A residue in oligo 2 was changed to a T residue in oligo 6, we concluded that the deletion–ligation product band, named A_T (Figure 3d), consisted of a mixture of products in which either the A or the T residue was present at this position. This observation, together with the retention of the 5′ label of oligo 1 in the A_T band, indicates that the deletion–ligation may occur at various locations within the 7 nucleotide direct repeat sequences, but this only becomes apparent with these mismatched oligos because of the presence of the single alteration in this direct repeat.

With this particular mated cell extract, the major product from oligo 1 plus 2 was always A, but in some experiments (for example, in Figure 7c), product D replaced products B plus C, even though the molar ratio of complementary oligos was 1:1. Oligos 2 plus 5 gave products A and B while the control reaction run in parallel with oligos 2 plus 1 gave products A and D (Figures 7c and 3c). Therefore, even though the single base change in oligo 5 did not alter

the direct repeats that border the sequences deleted to form products A, B, or D, it was sufficient to alter the distribution of products in the experiment shown in Figure 7c.

Oligos 2 plus 3 were also tested to determine the effect of a two nucleotide complementary overhang in the 2–3 monomer duplex and a 2 nucleotide insertion in the putative oligo 2–1 dimer (Figure 7c, lanes 3 and 4). Interestingly, although deletion–ligation products were formed efficiently from oligos 2 and 3 (Figure 7c, lane 3), the 5′ label in input oligo 3 was not retained in any deletion–ligation product (lane 4). The oligo 2 plus 3 reactions each gave products A and B (Figures 3b and 3c). It is interesting to note that the two base overhang present in the oligo 2 plus 3 duplex allows product formation, while the four base overhang in oligos 3 plus 4 does not.

The specificity of the reaction was further tested by using several additional pairs of complementary oligos (Figure 1). Oligos 7–14 were similar to oligos 1 or 2, but had various internal single base changes and short terminal truncations or additions. None of these efficiently formed A–E, or any other detectable products longer than the input substrate oligonucleotides (Figures 7d and e). Oligos 21 and 22, a pair of AT-rich oligos of unrelated sequence (Figure 1), were also inactive as substrates for formation of deletion–ligation products longer than the input oligos (data not shown). In all the above experiments, parallel reactions were performed with oligos 1 and 2 as positive controls.

Taken together, these data indicate that, as in vivo, the in vitro deletion–ligation reaction has a high degree of sequence specificity.

Discussion

The Deletion–Ligation Activity Has the Properties Expected for the Activity That Rearranges the Genome In Vivo

We have shown here that extracts of mated Tetrahymena cells contain a novel enzymatic activity capable of catalyzing a specific set of deletion–ligation reactions using synthetic DNA oligonucleotide substrates. Several observations regarding this in vitro reaction suggest that it could be catalyzed by the activity responsible for the multiple deletion–ligation reactions that occur in vivo in the developing macronuclei of ciliates.

First, the sequence specificity and features of the in vitro deletion–ligation reaction resemble those of the in vivo deletion–ligations. In both cases the sequence between a pair of short direct repeats is deleted along with one direct repeat sequence. Furthermore, in Tetrahymena, small variations in the exact point of ligation occur in vivo (Austerberry and Yao, 1987); in vitro, products A, B, and C, for example, appear to use overlapping repeats and resemble this type of variability. On a larger scale, different IES boundary repeats appear to be alternatively used in vivo (Austerberry et al., 1984; Howard and Blackburn, 1985; White and Allen, 1986); this difference is paralleled in the case of different overlapping sets of repeats being used in products A, B, and C versus D and E. The in vivo IESs that have been sequenced are AT-rich (Klobutcher et al., 1984; Ribas-Aparicio et al., 1987; Herrick et al., 1987; Austerberry and Yao, 1987; Forney and Blackburn, 1988). In the in vitro reaction, although GC-rich regions were present in the substrate oligos, the sequence deleted was invariably AT-rich.

Second, the specific deletion–ligation activity is greatly enriched in cells undergoing macronuclear development compared with nonmated starved or vegetatively growing cells. Since starvation may serve to induce production of enzymes required for mating, the presence of a low amount of deletion–ligation activity in starved cells is not unexpected.

The efficiency of the reaction in vitro is high, and is comparable to the levels of activity that might be expected for enzymes that carry out the deletion–ligation events in macronuclear development. The measurement of the percent conversion of the input oligo 2 to product A (0.3%) gives a calculated rate of $\sim 10^5$ molecules of product A produced per min per cell equivalent with initial 1 μM concentrations of oligo 1 and oligo 2. It has been estimated that there are about 6000 deletion–ligation events per haploid genome (Yao et al., 1984) in less than 4 hr during macronuclear development in T. thermophila (Austerberry et al., 1984). With the macronuclear ploidy less than 8°C at this stage (Allis et al., 1987), each developing macronucleus would carry out an estimated maximum of $\sim 5 \times 10^4$ such events in this period. Therefore, the activity we find is present at levels more than sufficient to carry out the deletions observed in vivo.

An intriguing finding was the complete inactivation of the deletion–ligation activity by brief exposure of extracts to temperatures of 38°C or higher prior to reaction at 30°C. This contrasted with other activities, such as general ligation to form dimers, which were not affected (data not shown). Strikingly, this temperature inactivation profile exactly parallels the high temperature-induced lethality specific for Tetrahymena cells in the process of developing new macronuclei. Scholnick and Bruns (1982) showed that this sensitivity occurs within a specific time window during conjugation; we note that this time window includes the time period during which macronuclear genomic rearrangements, observed by molecular studies, take place in vivo (Austerberry et al., 1984; reviewed in Karrer, 1986). Our Tetrahymena extracts were made during this same time period.

Possible Mechanism of the In Vitro Deletion–Ligation Reaction

A significant property of the in vitro deletion–ligation reaction we have discovered is that a remarkably limited set of products is formed, whose common feature is a sequence predicted from deletion of DNA between a pair of direct repeats in a putative dimer intermediate. Such deletions invariably include one of the direct repeats.

Taken together, several findings suggest that a required intermediate is the specific dimer of oligo 1 and 2 shown in Figure 3: first, the internalization of the 5′ ^{32}P end-label of oligo 1 in product A; second, the elimination of labeled product A, B, and C formation when 5′ end–labeled oligo 2 was blocked at its 3′ end with a dideoxyadenylate resi-

due; third, the specific formation of the phosphatase resistant 1+1 band from 5′ end–labeled oligo 1 (Figures 2d and 7a), but not from 5′ end–labeled oligo 2. In addition, although sequence data indicate that a mixture of different end-to-end ligated dimers is formed, it is only the oligo 2–oligo 1 dimer that is used in these reactions. An increase in the amount of dimer band can be seen in fractions that eluted after the peak of deletion–ligation activity (fractions 90 and 100, Figure 6a). It is possible that this increase is due to an accumulation of the dimer in the absence of the deletion–ligation activity, which, when present in the active fractions, converts the oligo 2–1 dimers in this dimer mixture into products.

That the deletion–ligation does not involve general nonspecific ligation of degradation products is suggested by the separation of the deletion–ligation activity from general ligation of oligonucleotide substrates to form dimers (see Figures 5–7). Possible precursors of products A–E were not preferred nuclease breakdown products in any experiments. Furthermore, if products A–E were simply the products of ligation of preferred nuclease breakdown products, the frequency and sequence of the deletion–ligation products should be changed by changing the relative abundance of nuclease breakdown products. However, this was not the case when the breakdown product spectrum was altered by gel filtration or ion exchange chromatography of the activity, which partially removed nuclease activities (data not shown).

Various possible mechanisms could be envisioned for the deletion–ligation reaction. The activity has the physical properties of a protein enzyme, with no nuclease-sensitive component. The ATP requirement is consistent with an initial ligation step to produce the specific oligo 2–1 dimer, but ATP could also be required for other steps in the reaction such as the ligation following deletion. One possibility is that formation of the specific oligo 2–1 dimer is catalyzed by the same activity that catalyzes the ligation of the oligos following deletion.

Several features of the deletion–ligation reaction are inconsistent with a general homologous recombination mechanism. A possible pathway involving unequal crossover between the direct repeats on two different duplex dimer molecules of oligos 1 and 2 (Figure 4a) was ruled out for at least the major product, product A, by the double end–labeling experiment (Figure 4b). Furthermore, even though products B and E are complementary, they were largely formed as alternative products under different conditions (Figures 7a and 7c), and not concomitantly, as would be expected if they were simultaneously formed by homologous recombination. In addition, there were several other direct repeats, in dimers of oligos 1 and 2 in various orientations, that were not used even though the products predicted by their sequences would have been readily detectable in our assay. Finally, several of the oligo pairs tested with different sequences were not used as substrates for such product formation, even though their comparable dimers also contain direct repeats.

We also considered a variant on a general recombination mechanism: formation of a structure resembling a Holliday intermediate, by melting and intramolecular cruciform formation within a single duplex of oligos 1 and 2, and action on it by the enzymes that carry out the cutting and ligation at the Holliday junction in general recombination. Although the AT-rich sequences of oligos 1 and 2 contain various short inverted repeats, allowing such hypothetical Holliday intermediates to be drawn for B, C, and E, comparable structures cannot be drawn to generate the major products A and D in an analogous way. In addition, branch migration of such intermediates predicts formation of many additional possible products that are not seen. Finally, involvement of such an intermediate does not predict the observed inability to form either products B and C when the 3′ end of oligo 2 was blocked (Figure 4c), or products A, B, and C from oligo 3 and 4 substrates (Figure 7b).

We propose that the in vitro deletion–ligation reaction occurs by the type of intramolecular mechanism diagrammed in Figure 4a. Such a general scheme was proposed by Ribas-Aparicio et al. (1987) for IES removal in vivo. The AT-rich character of the deleted sequences suggests that they are unpaired in at least one step in the reaction. Such a single-stranded looping-out of the IES could bring its bordering nucleotides together for ligation. As suggested by Ribas-Aparicio et al. (1987), the inverted repeats that commonly are located at the ends of IESs could aid in this placement of the flanking nucleotides. Inverted repeats ranging from 5–7 nucleotides are present between or overlapping the direct repeats involved in the production of products A–E. In vivo, deletion–ligation could occur on one strand only, as we observe in the in vitro reactions, with DNA synthesis copying the deleted strand to give the final double-stranded deleted macronuclear product.

Even with the 40 nucleotide oligo substrates I1 and I2 containing extended flanking O. nova sequences, no product resulting from the deletion of the 14 nucleotide IES of O. nova was formed with an efficiency comparable to the formation of products A, B, and C. This could reflect a difference in the action of the Tetrahymena activity under our reaction conditions, and possibly in vivo. In vivo, deletions of Tetrahymena IESs are characterized by microheterogeneity not seen in the Oxytricha counterparts. Clearly, as demonstrated above, while direct repeats are apparently always involved in the specific deletion–ligation reaction in vitro, they are not sufficient to specify product formation. The high specificity of the in vitro deletion–ligation suggests either that we chose substrates that serendipitously contained the necessary sequence and structural elements for deletion–ligation by Tetrahymena, or that there is general conservation of these elements among ciliates, even though they are processed differently by different ciliates.

Further research will be necessary to determine the role in ciliate macronuclear development of the highly specific deletion–ligation activity reported here and to understand its relationship to DNA processing in other contexts. We note that phenomena once thought to be confined to ciliated protozoans have been shown to have far-reaching implications which have enhanced our understanding of certain molecular processes. For example, the self-splic-

ing catalytic RNA of Tetrahymena and the structure and synthesis of ciliate telomeres are now known to have general biological significance (reviewed by Cech and Bass, 1986; Greider and Blackburn, 1989). We anticipate that an understanding of the mechanism of the unusual deletion–ligation reaction of ciliates may provide insights into DNA rearrangement mechanisms in other systems.

Experimental Procedures

Extract Preparation

Tetrahymena strains SB210 and PB9R (provided by E. Orias) were grown and mated as previously described (Greider and Blackburn, 1985), except that mating cells were not refed before preparation of extracts. Mated cells were extracted 12 hr after mating was initiated by mixing. Cells were spun down, washed with Dryl's solution (1.7 mM sodium citrate, 1.2 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 μM $CaCl_2$), the pellets were mixed with 5 vol of TMG–NaCl (10 mM Tris–HCl [pH 8.0], 1 mM $MgCl_2$, 10% glycerol, 10 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM Pepstatin, and 0.1 M NaCl), and RNasin (Promega) was added to 10 U/ml. Cells were lysed by the addition of 1/10 volume of 2% NP40 in TMG–NaCl with constant stirring at 4°C for 30 min. The lysate was centrifuged in an SW41 rotor at 30K rpm (100 × g) for 60 min at 4°C. Supernatants were fractionated on a Bio-Gel A-5m sizing column (Bio-Rad), and fractions possessing deletion–ligation activity were used in the experiments. Extracts were stored under liquid nitrogen.

Radiolabeling

DNA oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer and purified by polyacrylamide gel electrophoresis. 5′ end-labeling with [γ-^{32}P] ATP (7000 Ci/mmol; ICN) was performed by the procedure of Zarbl et al. (1985). Oligos were labeled at the 3′ end with 5′ [α-^{32}P]ddATP (3000 Ci/mmol; Amersham) by mixing 3 pmol 5′ [α-^{32}P]ddATP, 10 pmol oligo, and 48 U terminal deoxyribonucleotidyl transferase (IBI) in cobalt buffer (IBI) and incubating at 37°C for 30 min. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM. The radiolabeled oligos were separated from unincorporated triphosphates by running the reaction mixture on a C_{18} column (Waters Associates) and eluting with 50% methanol.

In Vitro Reactions

To assay for deletion and rejoining activity, 3–5 ng radiolabeled oligonucleotide per reaction was lyophilized to dryness and resuspended in 9 μl of a solution containing 50 μg/ml of the same oligo, unlabeled, and 50 μg/ml of the unlabeled complementary oligo. Complementary strands were annealed by heating to 90°C and incubating at 37°C for at least 15 min. The annealed oligos were then mixed with an ATP-regenerating system and Tetrahymena extract to yield a reaction mixture containing 3% PEG 6000, 1 mM spermidine, 0.1 mM spermine, 4 mM ATP, 10 mM phosphoenolpyruvate, 60 μg/ml pyruvate kinase (Boehringer Mannheim), 42 mM HEPES (pH 7.8), 40 mM KOAc, 7 mM $MgCl_2$, 0.1 mM EGTA, 0.8 mM DTT, and 20 μl extract in a final volume of 50 μl. The extract was made 0.1 mM in PMSF prior to addition to the reaction mixture, and was added to the mixture last to initiate the reaction. Samples were incubated at 30°C for 20 min. The reaction was stopped by the addition of 2 vol of 10 mM EDTA, 0.1 mg/ml tRNA, and 0.2 mg/ml proteinase K in TE (10 mM Tris, pH 7.5, 1 mM EDTA), followed by incubation at 37°C for at least 1 hour. Samples were extracted with an equal volume of phenol:chloroform, and the aqueous phase was precipitated in 0.3 M NaOAc and 95% ethanol.

Gel Electrophoresis

Pellets were resuspended in a loading buffer consisting of 90% formamide, 1× TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA [pH 8.0]), and 0.02% xylene cyanol, and were run on 40 cm 12% polyacylamide 8 M urea sequencing gels. The running buffer was 0.6× TBE. Gels were run at 1500–1800 V for approximately 3 hr, until the xylene cyanol FF dye had migrated 20 cm. Following electrophoresis, gels were dried onto Whatman 3MM paper and autoradiographed at −80°C with a Dupont Cronex Lightning-Plus intensifying screen.

Quantitative Analysis of Reaction Products

A standard IES reaction was performed at 30°C for 20 min with 5′ end–labeled oligo 2. Products were phenol extracted, ethanol precipitated, and run on a 12% polyacrylamide sequencing gel. Bands corresponding to the reaction products and the input band were cut out of each lane, emersed in Omnifluor (Du Pont)–toluene scintillation fluid, and the radioactivity counted. A patch of gel lane with no products was taken as background and subtracted from all measurements.

Phosphatase Treatment

Following ethanol precipitation, samples were resuspended in H_2O. One-third of the total volume was mixed with 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim), 50 mM Tris (pH 9.0), 1 mM $MgCl_2$, and 1 mM spermidine (Maniatis, et al., 1982) and incubated at 37°C for 30 min. Reactions were stopped by the addition of one-tenth volume of 200 mM EDTA, and samples were precipitated in 0.3 M NaOAc and 95% ethanol.

Isolation of Deletion–Ligation Products for Sequencing

Preparative polyacrylamide gels were run as described above, but were not dried down prior to autoradiography. Using the autoradiograms as a guide, bands were excised from the gels and eluted in 100 μl TE with 10 μg tRNA at 37°C for 1 hr. The eluate was removed from the gel fragments, mixed with 1 μg calf thymus DNA and ethanol precipitated. The DNA pellet obtained was resuspended in H_2O for sequencing according to the method of Maxam and Gilbert (1980).

Blotting and Hybridization

Products run on polycrylamide gels were blotted onto Nytran filters (Schleicher & Schuell) using an IBI model HBS Electroblotter. Blotting was conducted at 25 V, 0.35 amps at 4°C overnight. The running buffer was 0.6× TBE. Filters were baked under vacuum at 80°C for 2 hr. Filters were prehybridized and hybridized at 37°C using the conditions described by White et al. (1988), except that 0.5 mg/ml fish sperm DNA was used in place of the 1 mg/ml bovine serum albumin. The probe was oligo P2 5′ end–labeled with [γ-^{32}P]ATP as described above. Filters were washed in 2× SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, H_2O, 2 mM Na_2EDTA, 16 mM NaOH), 0.1% SDS (White et al., 1988), and autoradiographed as described above.

Preincubation Experiments

Extracts thawed from storage in liquid nitrogen were mixed with a cocktail containing all the components of the reaction except the oligonucleotide substrates, and incubated for 20 min under the conditions to be tested. Reactions were then initiated by the addition of oligonucleotides which had been radiolabeled and annealed as described above. Reactions were conducted at 30°C for 20 min. For testing the effect on activity of preincubation at different temperatures, the preincubation mixes were held at the stated temperature for 20 min, then kept on ice for no longer than 1 hr before adding oligonucleotides to initiate the reaction. Test preincubations with nucleases were done using the same procedure, but with addition to aliquots of the preincubation mix of either RNAase A at different concentrations (1–100 μg/ml final concentration), or micrococcal nuclease (3 or 30 U/ml final concentration) plus Ca^{2+} to 1 mM final concentration. Control preincubation reactions contained appropriate inhibitors that were added prior to nuclease addition: RNasin (120 U; Promega) for the RNAase A reactions, and EGTA to 2.5 mM for the micrococcal nuclease reactions. Inhibitors were added to the test reactions after preincubation.

Acknowledgments

We thank Nicholas Cozzarelli and Hatch Echols for stimulating and helpful discussions, and Monica Flessel, Vicki Lundblad, Claire Wyman, and Guo-Liang Yu for critical reading of the manuscript. This work was supported by grant GM 26259 from the National Institutes of Health to E. H. B.

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Received February 13, 1989; revised June 29, 1989.

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