Sequence microheterogeneity is generated at junctions of programmed DNA deletions in *Tetrahymena thermophila*

Charles F.Austerberry*, Richard Owen Snyder+ and Meng-Chao Yao

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104, USA

Received June 20, 1989; Revised and Accepted August 17, 1989

ABSTRACT

Regulated DNA deletions are known to occur to thousands of specific DNA segments in Tetrahymena during macronuclear development. In this study we determined the precision of this event by examining the junction sequences produced by three different deletions in many independent caryonidal lines. 0.9 kb deletions in region M produce at least 3 types of junction sequences, of which two have been determined and found to be different by 4 bp. The alternative 0.6 kb deletions in this region are much less variable. 1.1 kb deletions in region R, known from a previous study to be slightly variable, produce two types of junction sequences which are different from each other by 3 bp. Thus, developmentally regulated deletions in Tetrahymena can produce sequence microheterogeneity at their junctions. This process contributes significantly to the diversification of Tetrahymena's somatic genome.

INTRODUCTION

Programmed DNA rearrangements are known to occur during cellular differentiation, and may play significant roles in gene regulation (reviewed in ref. 1). One type of these rearrangements, internal DNA deletion, occurs to remarkable extents in ciliated protozoa (reviewed in ref. 2,3). For instance, in Tetrahymena thermophila more than five thousand defined segments of DNA are deleted in a specific 2-hour period during somatic nuclear (macronuclear) differentiation (4,5). The biological functions of these deletions are not clear, although in another ciliate (Oxytricha) some deletions actually lead to the joining of interrupted open reading frames (6,7,8).

One distinct feature of DNA deletion in ciliates is its apparent precision. Most deletions occur uniformly in different macronuclei, producing DNAs which appear to be identical in structure as judged by restriction mapping. Although some deletions in Tetrahymena are known to generate more than one type of product, such alternative products are limited in number, each having a defined structure which is different from the others by several hundred bp. (4,9,10). Thus, both the alternative and the uniform deletions are highly regulated. Nucleotide sequence analysis has not revealed any feature which may provide a convenient explanation for this regulation. The only common feature found is a pair of short direct repeats (1−8 bp) flanking both ends of the deleted region (8,11).

Although DNA deletion in ciliates appears to be precise as measured by restriction mapping, this method can not detect variations consisting of a few bp. Whether a deletion is indeed precise at the nucleotide level is critical for understanding its mechanism and function. The first hint that a deletion can be imprecise was derived from an earlier study in which we found that one junction sequence produced by a uniform deletion in region

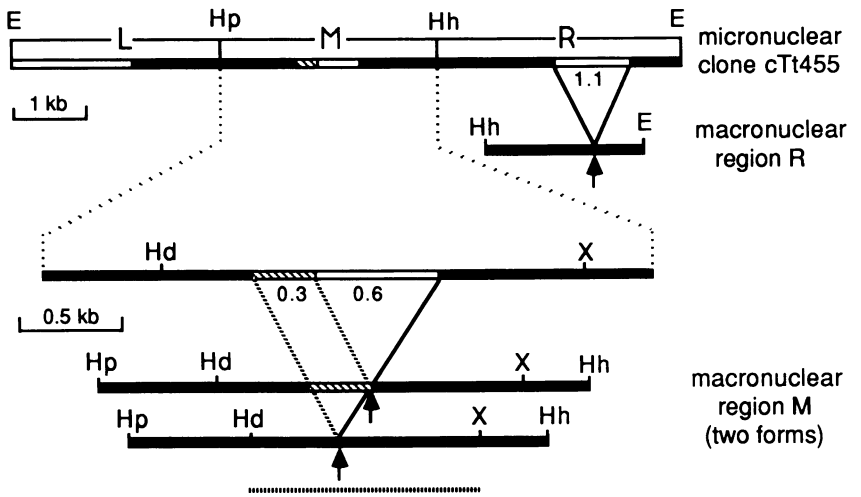

Figure 1. *DNA Deletion in Regions M and R.* The 9.5 kb *Eco*RI (E) fragment of micronuclear DNA in clone cTt455 contains unique *Hpa*II (Hp) and *Hha*I (Hh) sites defined as boundaries of regions L, M, and R as shown. Each region contains micronucleus-specific DNA (open bars) that is normally deleted during macronuclear development, as well as DNA that is retained in the macronucleus (solid bars). Region M also contains a 0.3 kb segment of alternatively deleted DNA (hatched bar). The sizes of the deleted segments are given in Kb. The alternative junctions in region M and one junction in region R resulting from these deletions are indicated (arrows). The *Hind*III (Hd) and *Xba*I (X) sites are shown only on the large-scale maps of region M. The broken horizontal line indicates the 1.2 Kb *Hind*III–*Xba*I fragment used as a hybridization probe in Fig. 3a. Additional *Hind*III sites within and to the right of region R (not shown) allow the generation of discrete fragments containing junctions created by 0.6 or 0.9 Kb deletions in region M (2.2 or 1.9 Kb *Hind*III fragments, respectively) and by 1.1 Kb deletions in region R (2.8 Kb *Hind*III fragments).

R is also present in most, but not all, of the macronuclear region R DNAs from 40 cell lines analyzed by oligonucleotide hybridization (12).

In this study the precisions of two alternative deletions in a neighboring region (region M) as well as the deletion in region R were further investigated by oligonucleotide hybridization and sequencing. We found that each of these three deletions occurs with a different degree of precision at the nucleotide level, and that heterogenieity at this level is caused by shifting of deletion endpoints by 3 or 4 bp.

MATERIALS AND METHODS

Tetrahymena Strains

Strains B1868-IV, CU427, and CU428 were generously provided by P. Bruns, Cornell University, Ithaca, N.Y. Strain A*III was generously provided by D. Nanney, University of Illinois, Urbana. Caryonidal lines were obtained from normal and genomic exclusion matings as previously described (12). Caryonide 427-1a was grown vegetatively for approximately 40 fissions to allow allelic assortment to occur, and several subcaryonidal lines were established from randomly selected individual cells.

DNA Clones

Recombinant λ phage clones cTt455 and cTt1110 contain micronuclear and macronuclear versions, respectively, of the same EcoR1 fragment from *T. thermophila* strain B1868-IV,

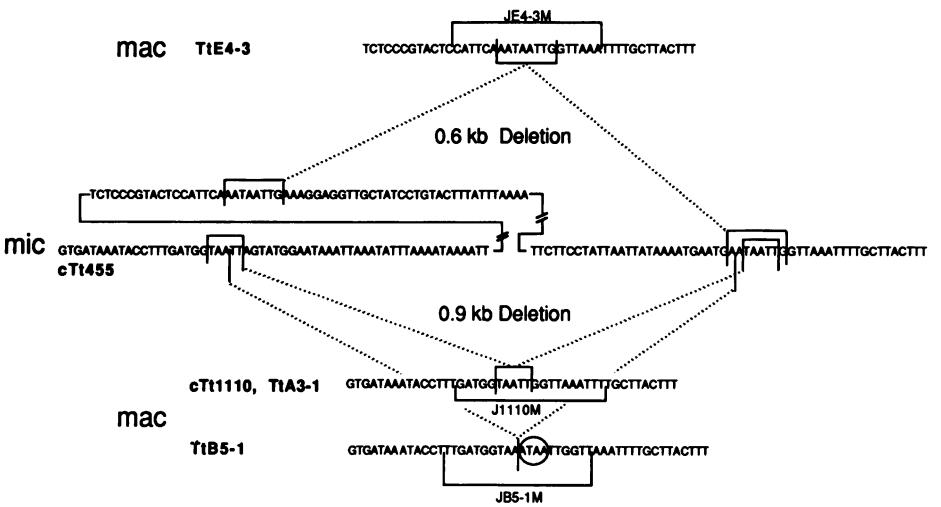

Figure 2. *Nucleotide Sequences of Junctions Created by Deletions in Region M.* Sequences near to and including the deletion junctions of region M are shown as found in micronuclear clone cTt455 and in macronuclear clones TtE4-3, cT1110, TtA3-1, and TtB5-1. The macronuclear sequences are derived from the micronuclear sequence by 0.6 kb or 0.9 kb deletions (broken lines) during macronuclear development. Endpoints of the 0.6 kb deletion (as in clone TtE4-3) or one type of 0.9 kb deletion (as in clones cTt1110 and TtA3-1) are short direct repeats (enclosed by unlabeled brackets) of which one copy is retained in the macronuclear sequences. Endpoints of another type of 0.9 kb deletion (as in clone TtB5-1) are indicated by vertical lines. Four nucleotides at the deletion junction in TtB5-1 (circled) are absent from cTt1110 and TtA3-1 because of the different endpoints involved in these 0.9 kb deletions. Labeled brackets indicate the sequences of synthetic oligonucleotide probes used to assess the consistency of the deletion process at the nucleotide level.

and have been previously described (5,12). Three additional clones were obtained, each containing the same EcoR1 fragment (macronuclear version) from a different subcaryonidal line derived from caryonide 427-la (see above). All of the various region R and region M deletion junctions detected in the macronuclear genome of the caryonidal line are present in greater abundance in one or more of the three subcaryonidal lines. Partial libraries were constructed by ligating size-selected EcoRI fragments of whole-cell Tetrahymena DNAs from the subcaryonidal lines to λ gtWES-EcoRI arms (Bethesda Research Laboratories, Gaithersburg, MD). Recombinant phages containing the desired EcoRI fragment were identified and isolated as previously described (5). The inserts were analyzed by Southern (13) hybridizations using nick-translated DNA fragments and synthetic oligonucleotides as probes. In all cases, the inserts faithfully represented the macronuclear genomes of their respective subcaryonidal lines. *Hind*III-XbaI fragments of phage clones from each of the three libraries were subcloned in plasmids pEMBL18+ and pEMBL18− (gifts of G. Herrick, University of Utah, Salt Lake City). Plasmid subclones containing deletion junctions from region R or M were analyzed by sequencing.

DNA Sequencing

Single-stranded templates were prepared and sequenced as previously described (12). The universal 17-mer sequencing primer (New England Biolabs, Beverly, MA, cat. # 1211) as well as specific primers (made on Applied Biosystems automatic DNA synthesizers) matching determined Tetrahymena sequences were used.

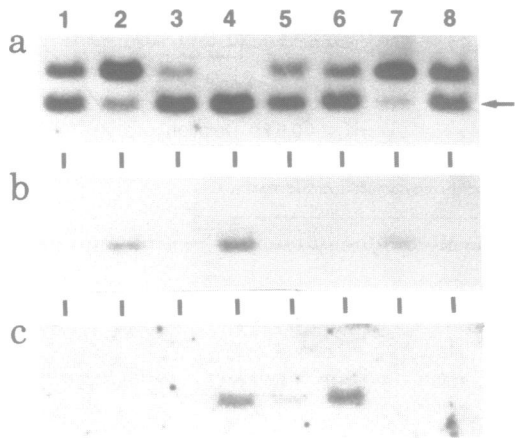

Figure 3. *Oligonucleotide Hybridization Analysis of Junction Sequences Created by 0.9 kb DNA Deletions in Region M.* Southern blot of DNAs from caryonidal lines obtained from Round II of a CU427×A*III genomic exclusion mating (lanes 1–4, caryonides 2a–2d; lanes 5–8, caryonides 5a–5d) hybridized sequentially with oligonucleotide probe J1110M(panel b), polynucleotide region M probe (panel a), and oligonucleotide probe JB5-1M (panel c). The oligonucleotide probes (panels b and c) hybridized with the 1.9 kb *Hind*III fragment (arrow, panel a) obtained from region M DNA rearranged by 0.9 kb deletion during macronuclear development (Fig. 1 and refs. 4,11).

Southern Hybridizations

2–5 μg of whole-cell DNAs from vegetative cultures of caryonidal strains were completely digested with *Hind*III, fractionated by agarose gel electrophoresis, and blotted onto nylon filters (13). To analyze the precise sequence at the rejoined junctions created by deletion, oligonucleotides were used as hybridization probes. The 20-mers J1110M, JB5-1M, JE4-3M, and JA3-1R and the 24-mer J1110R (see Results) were made on Applied Biosystems DNA synthesizers. They were ^{32}P-labeled at the 5′ end with T4 polynucleotide kinase (14). Hybridizations with oligonucleotide probes using approximately 107 dpm per ml were performed as previously described (12) or by using the method of Wallace and

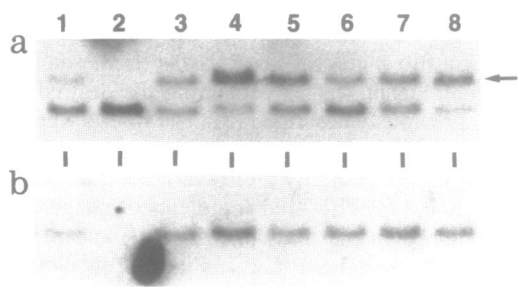

Figure 4. *Oligonucleotide Hybridization Analysis of Junction Sequences Created by 0.6 kb DNA Deletions in Region M.* Southern blot of CU427 × A*III RdII caryonidal DNAs (lanes 1–4, caryonides 1a–1d; lanes 5–8, caryonides 4a–4d) hybridized with polynucleotide region M probe (panel a) and oligonucleotide probe JE4-3M (panel b). The oligonucleotide probe used in panel b hybridized with the 2.2 kb *Hind*III fragment (arrow, panel a) obtained from region M DNA rearranged by 0.6 kb deletion during macronuclear development (Fig. 1 and refs 4,11).

Miyada (15). Hybridizations with a nick-translated polynucleotide probe, a 1.2 kb *Hind*III-XbaI fragment containing the region M deletion junction (Fig. 1), were as previously described (12,13). Probes were completely removed by high stringency washes prior to reuse of filters.

RESULTS

Analysis of DNA Deletion in Region M

Nucleotide Sequence Consistency of Deletion Junctions in Region M. During macronuclear development two alternative deletions of either 0.9 or 0.6 kb occur at roughly equal frequencies in region M of Tetrahymena (4,11) (Fig. 1). Nucleotide sequences of resulting junctions, one produced by each of these two alternative deletions, have been previously determined (11). In order to characterize the consistency of these deletions at the nucleotide level, synthetic 20-mer oligodeoxy ribonucleotides (J1110M and JE4-3M) matching these two junction sequences (Fig. 2) were prepared and used as probes in Southern (13) hybridizations of *Hind*III-digested whole-cell DNAs from caryonidal lines containing one or both of the alternative macronuclear forms of region M.

DNAs from 35 caryonidal lines of *T. thermophila* known to yield the 1.9 kb *Hind*III fragment resulting from 0.9 kb deletion were hybridized with the oligonucleotide (J1110M) matching the previously determined 0.9 kb deletion junction sequence in λ phage clone cTt1110 (11). Under stringent conditions (posthybridization wash in 0.75×SSPE at 37°C), DNAs from 26 of these 35 caryonides hybridized with J1110M. Results for eight caryonides from a CU427×A*III genomic exclusion mating are shown in Fig. 3. Only three of these DNAs hybridized with J1110M (Fig. 3b, lanes 2,4 and 7). Incubation of the same filter with a polynucleotide probe for region M (see Fig. 1) confirmed that the five DNAs not hybridizing with J1110M nonetheless contained significant levels of the 1.9 kb *Hind*III fragment produced by 0.9 kb deletion (arrow, Fig. 3a). Junctions created by the 0.9 kb deletion in region M, therefore, must be heterogeneous at the nucleotide level.

DNAs from 19 caryonidal lines known to yield the 2.2 kb *Hind*III fragment resulting from 0.6 kb deletion in region M were hybridized with the synthetic oligonucleotide (JE4-3M) matching the previously determined 0.6 kb deletion junction sequence in 3l phage clone TtE4-3 (11). Under stringent conditions, all 19 hybridized with the probe (Fig. 4, and data not shown), and the intensity of the hybridization with this probe (Fig. 4b) did not differ significantly from that seen with the larger nick-translated probe (Fig. 4a). This result suggests that nearly all of the 0.6 kb deletion junctions in these caryonides contain the same sequence present in the oligonucleotide probe. Thus, the 0.6 kb deletion in region M appears to be highly reproducible in independently developed nuclei. Nucleotide Sequence of the Variant Junction in Region M. To determine the structure of the 0.9 kb deletion junctions which failed to hybridize with the oligonucleotide probe J1110M, such a junction was cloned in λ phage and its nucleotide sequence was determined. The 3l clone TtB5-1 contains region M DNA from the subcaryonidal line 427-1a-8 of *T. thermophila*; both the genomic and the cloned DNAs failed to hybridize with the oligonucleotide probe J1110M (data not shown). The nucleotide sequence of this non-hybridizing junction is shown in Fig. 2. This sequence is identical to that previously determined (11) in clone cTt1110; except for an additional 4 bp located precisely at the deletion junction. This new sequence is created by a deletion starting at a specific position within the left copy of the 5 bp direct repeats which flank the two ends of the original deletion, and terminating one bp left of the right copy of this repeat (Fig. 2); part of the left repeat and all of the right repeat

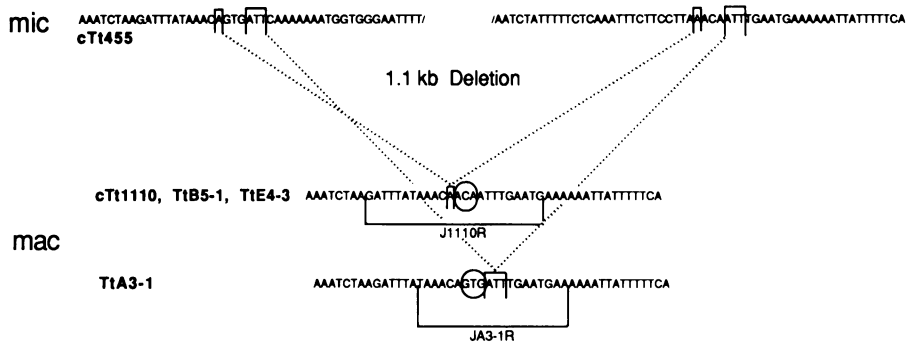

Figure 5. *Nucleotide Sequences of Junctions Created by Deletions in Region R.* Sequences near to and including the deletion junctions of region R are shown as found in micronuclear clone cTt455 and in macronuclear clones cTt1110, TtB5-1, TtE4-3, and TtA3-1. The macronuclear sequences are derived from the micronuclear sequence by two types of 1.1 kb deletions (broken lines) during macronuclear development. In both types of deletions the endpoints are short direct repeats (enclosed by unlabeled brackets) of which one copy is retained in the macronuclear sequences. Three nucleotides (circled) of the junction sequence in clone TtA3-1 differ from those in clones cTt1110, TtB5-1, and TtE4-3 because of the different deletion endpoints involved. Labeled brackets indicate the sequences of synthetic oligonucleotide probes used to assess the consistency of the deletion process at the nucleotide level.

are therefore retained in this macronucleus. Thus, unlike any other deletion known in ciliates, this deletion is not flanked by direct repeats.

We have also determined the 0.9 kb deletion junction sequence in another 3l clone, TtA3-1, from the subcaryonidal line 427-1a-2 which did hybridize with the oligonucleotide probe J1110M. As expected, the sequence (total of 195 bp were determined) is identical to that found in clone cTt1110. This result confirms the oligonucleotide hybridization data, and suggests that no other variation in sequence occurs in this region.

Occurrence of the New Junction Sequence in Region M. DNAs from 20 caryonidal lines containing region M junctions of the 0.9 kb deletion, three of which hybridized with the oligonucleotide probe J1110M, were tested with an oligonucleotide probe matching the

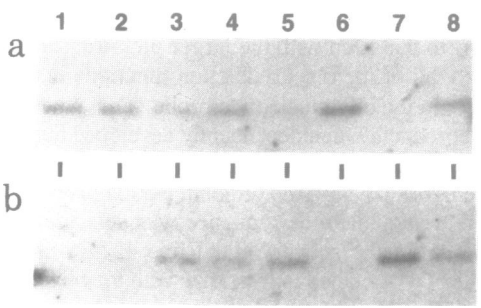

Figure 6. *Oligonucleotide Hybridization Analysis of Junction Sequences Created by Deletions in Region R.* Southern blot of CV428 × A*III RdIII caryonidal DNAs (lanes 1−4, caryonides 3a−3d; lanes 5−8, caryonides 4a−4d) hybridized with oligonucleotide probe J1110R (panel a) and oligonucleotide probe JA3-1R (panel b). Both probes hybridized with the 2.8 kb *Hind*III fragment obtained from region R DNA rearranged by 1.1 kb deletion during macronuclear development (Fig. 1 and ref. 12).

new junction sequence in order to assess its frequency of occurrence, and to determine whether additional junction sequences are present. The oligonucleotide probe used (JB5-1M) is indicated in Fig. 2. It is specific for DNAs containing the new junction sequence, and does not hybridize with the previously determined junction sequence in clone cTt1110 (data not shown). It hybridized with some 0.9 kb deletion junctions in region M, but the pattern of hybridization was not always complementary to that of the original J1110M probe. Of the 20 caryonidal lines selected for analysis, DNAs from 7 hybridized with JB5-1M (Fig. 3c, and data not shown), one of which (Fig. 3, lane 4) also hybridized with the original probe J1110M. Significantly, some samples containing an abundance of products of 0.9 kb deletion hybridized with neither J1110M nor JB5-1M (e.g. lanes 1, 3, and 8 in Fig. 3). Thus, the two junction sequences of 0.9 kb deletion identified thus far are clearly not the only junction sequences created by this deletion.

Analysis of DNA Deletion in Region R

In a previous study we determined that the junction sequence created by deletion in region R in *T. thermophila* strain B1868IV and isolated in the λ phage clone cTt1110 is also present in 40 caryonidal lines (12). We have now expanded this study to include 32 additional caryonidal lines, of which 31 also contain this junction sequence. In addition to the one caryonidal line which failed to hybridize at all with the J1110R oligonucleotide (Fig. 6a, lane 7), several other caryonidal lines also contain varying proportions of non-hybridizing junction sequences as shown by assortment of such junctions among subcaryonidal lines (12). DNA containing a non-hybridizing junction from the subcaryonidal line 427-1a-2 was cloned (λ phage clone TtA3-1) and the R junction sequence determined. The sequence is identical to that in clone cTt1110 except for a 3 bp difference located precisely at the deletion junction (Fig. 5). This new junction is created by a deletion with its two ends located within a pair of 3-bp direct repeats (5′-ATT-3′) lying downstream of the 5′-TAAACA-3′ repeats which flank the previously determined deleted region seen in cTt1110 (Fig. 5); the precise location of the end points relative to the 5′-ATT-3′ repeat sequence must be the same on both sides of the deletion, because precisely one copy of the repeat is deleted.

The region R deletion junction sequences in two other clones (TtB5-1 and TtE4-3), both of which hybridized with the original region R oligonucleotide probe (J1110R), were also determined. As expected, they are identical to the previously determined region R junction sequence (Fig. 5). The surrounding sequences (>60 bp on both sides of the junction) are also identical. These results confirm the oligonucleotide hybridization results, and further indicate that no other sequence variation is produced near this deletion junction during development. To determine the distribution of the new region R junction sequence, DNAs from several caryonidal lines were selected for oligonucleotide hybridization based on their relatively low level of hybridization with the original junction sequence (J1110R). Of the 20 caryonidal lines tested, DNAs from 15 hybridized with the new junction sequence (JA3-1R) (Fig. 6b, and data not shown). The relative strengths with which the two probes hybridized are complementary, i.e. samples that failed to hybridize at all with one probe hybridized strongly with the other, and relatively weak hybridization with one probe is correlated with relatively strong hybridization with the other (Fig. 6). Superposition of the two autoradiographs shown in figure 6 produced composite bands of equal intensity in all 8 lanes (data not shown). This complementarity suggests that the two macronuclear region R junction sequences identified thus far may together constitute the majority, and possibly all, of the region R junction sequences present in these DNAs.

DISCUSSION

By oligonucleotide hybridization and DNA sequencing we have shown that DNA deletion in *Tetrahymena thermophila* can produce variable junction sequences. The extent of this variation differs in the three deletions studied and is greatest in the 0.9 kb deletion in region M. One junction sequence created by the 0.9 kb region M deletion is present in 26 of 35 lines analyzed and the other in 7 of 20 lines analyzed. While some lines contain both junction sequences, others clearly contain neither. Thus there is at least one more type of junction sequence produced by 0.9 kb deletion in region M which we have not yet determined. The variation in the deletion junction produced in region R is more limited. Again two junction sequences have been determined, one of which (J1110R) is clearly the most frequent because it is present in 71 of 72 lines analyzed, and in many of these lines it may account for most, if not all, of the region R junctions (12). The other junction sequence (JA3-1R) was found in 15 of 20 lines suspected to contain this sequence, often as a minor fraction (Fig. 6, and data not shown). Together these two sequences may account for most, and possibly all, of the junctions produced by deletion in region R. The 0.6 kb deletion in region M appears to be the most consistent. In all 19 lines analyzed the same junction sequence is present in high amounts. However, the sensitivity of the method does not exclude the possibility that small proportions (up to 1/4) of other junction sequences are present in some lines.

The variant junctions characterized by DNA sequencing differ from their respective non-hybridizing oligonucleotide probes by 3 or 4 bases. Junction sequences differing from the probes by only 1 or 2 bases are not likely present in significant amounts because they probably would have been identified as non-hybridizing variants. Previous studies in this (16,17,18) and other laboratories (reviewed in ref. 19) have shown that Southern hybridization assays using oligonucleotide probes can be sensitive to single-base internal mismatches. Final post-hybridization washes used in this study (e.g., 1 hr in 0.75× SSPE, 37°C for most 20-mers) were expected to dissociate duplexes with one central mismatched base because less stringent washes (1 hr in 2× SSPE, 37°C) were sufficient to completely dissociate the probes from the known variant junctions while slightly more stringent washes (e.g., 1 hr in 0.5×SSPE, 37°C) began to dissociate perfectly matched duplexes (unpublished observations).

The junctional diversity described here is distinct from the phenomenon of alternative deletion (4,9,10,11). The former involves variation of only a few bp, whereas alternative deletion produces differences of hundreds of bp. Both types of variation occur in region M, and yet a continuous range of intermediate-sized deletion products does not result. Thus, we consider alternative deletion and junctional diversity as separable events. This situation is analogous to the assembly of immunoglobin genes in B cells, in which alternative combinations of gene segments can be joined, but also junctions created using the same segments can vary by a few bp (reviewed in ref. 20). The junctional diversity we have observed in Tetrahymena can be fully explained by imprecision in the deletion process, analogous to 'junctional site diversity' in the assembly of immunoglobulin genes (reviewed in ref. 20). We have not observed template-independent insertion of nucleotides, such as the N regions observed in immunoglobulin genes (21).

The use of caryonidal lines allows us to conclude that the variations in junction sequences are not due to differences in genetic background of the cells examined. The four caryonidal lines derived from a mating pair contain macronuclei developed independently from the same germinal genome, analogous to the different somatic cells of an individual metazoan

organism, yet the junction sequences in them can be different. In addition, many of the lines used are produced through genomic exclusion mating, which results in the formation of homozygous genomes. Thus, allelic differences in the germ line can not be responsible for producing different junction sequences in the macronucleus. We thus conclude that the junction sequence variation is inherent in the deletion mechanism. It is the result of a developmental event which generates somatic DNA heterogeneity both within a clone and among clones with identical germline genomes. If similar variation also occurs in the thousands of other deletions in the Tetrahymena genome, the overall heterogeneity could be astronomical ($2^{4,000}$, if there are 4,000 sites each producing 2 different junctions). Since macronuclear alleles assort during vegetative growth, such a heterogeneity virtually ensures that no two somatic genomes from a single germline are alike. The impact of such a mechanism is unknown, and depends largely on the functions of the rearranged DNA. Although the two regions of DNA studied here have unknown function, it is possible that these or other deletions might have significant influences on cellular activities. An approximately 1.3 kb poly(A+) RNA appears to be transcribed from DNA lying between the region M and region R deletion junctions (M. Altschuler and M.-C. Yao, unpublished observations). The extremely biased base composition (approx. 80% A + T) of the sequences surrounding both junctions suggests that these deletions probably do not create or destroy open reading frames (11, 12). Whether these deletion junctions are transcribed remains to be determined.

Comparisons of the variant junction sequences offer some insight to the mechanism of deletion. In region R and region M both variant junctions can be produced by simple deletions without other complicated events. In region R the two junctions differ because of 3 bp shifts of both endpoints in the same direction; thus, the deleted segments are identical in length. In the 0.9 kb deletion of region M the two characterized junctions differ by 4 bp, and the endpoint shifts in this case are unequal. In both cases the variation is small, implying a well regulated mechanism which allows only limited degrees of variation to occur. This variation also shows that a deletion endpoint is not strictly associated with a particular terminal direct repeat. Thus, although terminal direct repeats are the most common feature found among deletion junctions in ciliates, their role may be limited. We suggest that the site specificity of a particular deletion is determined by some other sequence elements, such as the homopurine sequences flanking both region M deletions (11), but the precise joining points are influenced by the presence of short direct repeats. In this sense it is perhaps significant that the longer 8 bp repeat is associated with the more reproducible junction produced by the 0.6 kb deletion in region M and the shorter 5 bp repeat is associated with the more variable junction produced by the 0.9 kb deletion in region M.

ACKNOWLEDGEMENTS

This work was initiated in the Department of Biology, Washington University to partially fulfill the Ph.D. degree requirements of C.F.A. This work was supported by a Public Health Service grant from the National Institute of General Medical Sciences (GM 26210). M.-C.Y. was the recipient of a National Institutes of Health Research Career Award (HD 00547). C.F.A. was supported by a National Institutes of Health Predoctoral Training Grant (GM 07067) and a National Science Foundation Graduate Fellowship.

Present addresses: *Department of Biology, Creighton University, Omaha, NE 68178 and +Department of Microbiology, Health Sciences Center, SUNY at Stony Brook, Stony Brook, NY 11794, USA

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