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A developmentally regulated deletion element with long terminal repeats has *cis*-acting sequences in the flanking DNA

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Received October 26, 1999; Revised and Accepted January 24, 2000

ABSTRACT

Approximately 6000 specific DNA deletion events occur during development of the somatic macronucleus of the ciliate *Tetrahymena*. The eliminated Tlr1 element is 13 kb or more in length and has an 825 bp inverted repeat near the rearrangement junctions. A functional analysis of the *cis*-acting sequences required for Tlr1 rearrangement was performed. A construct consisting of the entire inverted repeat and several hundred base pairs of flanking DNA on each side was rearranged accurately *in vivo* and displayed junctional variability similar to the chromosomal Tlr1 rearrangement. Thus, 11 kb or more of internal element DNA is not required *in cis* for DNA rearrangement. A second construct with only 51 bp of *Tetrahymena* DNA flanking the right junction underwent aberrant rearrangement. Thus, a signal for determination of the Tlr1 junction is located in the flanking DNA, 51 bp or more from the right junction. Within the Tlr1 inverted repeat are 19 bp tandem repeats. A construct with the 19mer repeat region deleted from the right half of the inverted repeat utilized normal rearrangement junctions. Thus, despite its transposon-like structure, Tlr1 is similar to other DNA rearrangements in *Tetrahymena* in possessing *cis*-acting sequences outside the deleted DNA.

INTRODUCTION

During sexual reproduction in the ciliated protozoan, *Tetrahymena*, the somatic macronucleus develops from a mitotic product of the germ line micronucleus. Macronuclear development entails extensive DNA rearrangement resulting in a decrease in the DNA sequence complexity of ~10–20%. The remaining DNA is replicated to 45C, except rDNA which is amplified to ~10 000 copies per macronucleus (1). The vast majority of DNA rearrangements in *Tetrahymena* are breakage and rejoining events in which an internal sequence is eliminated and the flanking sequences are ligated (reviewed in 2). These events occur at an estimated 6000 different sites (3). At the majority of these sites, 0.5–3 kb of DNA is eliminated. However, longer

stretches of micronuclear-limited DNA of 10 kb or more have been cloned (4,5). The deletion events are highly regular in the sense that a given site undergoes rearrangement in every developing macronucleus. Some of these rearrangements utilize alternative junctions, resulting in a limited repertoire of rearrangement products from that site (6–8).

Nine of the deleted elements in *Tetrahymena*, including the M, R, Tlr1 and mse2.9 elements, have been described at the sequence level. The M rearrangement deletes 600 or 900 bp of micronuclear-limited DNA. The two alternative rearrangements utilize different left boundaries, M1 and M2, which are 300 bp apart in the micronuclear genome, and the same right boundary, M3 (6). *In vivo* analysis of M region constructs showed that a *cis*-acting sequence consisting of a 10 bp polypurine tract, is located in the flanking DNA near the rearrangement junctions (9). This sequence motif is sufficient to specify a cut site ~45 bp away in an orientation-specific manner. However, the polypurine tract has not been found near the boundaries of any of the other deleted elements that have been analyzed to date.

Two other elements have been shown to have *cis*-acting sequences in the flanking DNA, although in these cases the critical sequences are not as well defined. One of these, mse2.9, is a 2.9 kb DNA element in the second intron of a gene of unknown function (10). A 10 bp inverted repeat is present in the critical region, 70–80 bp from the junction sites (11). The R element, a short deleted element close to M, also has *cis*-acting sequences on both sides of the element. These are evidently complex in nature, because clusters of base substitutions throughout the 70 bp region that is critical for rearrangement did not affect rearrangement efficiency or accuracy. Furthermore, flanking sequences on the right can substitute for those on the left, even though there is no sequence homology between them (12).

Tlr1 belongs to the less common class of rearrangements that delete large amounts of DNA (4,5). The Tlr1 element is 13 kb or more in length (Fig. 1). Tlr1 rearrangement occurs at 10–12 h of mating, the same time as the deletion of the shorter elements (Capowski and Karrer, unpublished data).

A discrete array of alternate junctions are observed for Tlr1 (7,13). One junction is favored, and the joining at that junction produces the 'major' rearrangement product. The left boundary of Tlr1 ranges over 296 bp in the minor variants and

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the right boundary over 196 bp. The Tlr1 major rearrangement also displays junctional microheterogeneity over 6–7 bp (13).

The most striking structural feature of Tlr1 is an 825 bp inverted repeat near the rearrangement junctions. The outermost half of the inverted repeat contains tandem repeats of two different 19mers, 19A and 19B. In the case of the major rearrangement product, the entire inverted repeat on the right side is within eliminated DNA, whereas the outermost 245 bp of the repeat, including the 19A tandem repeats, is retained at the left.

Southern analysis indicated that 19A and 19B are associated with each other at six or seven sites in the micronuclear genome and that the 19mers occur in pairs of similar sized restriction fragments (7). No macronuclear fragments apart from the one containing the left boundary of Tlr1 were detected, implying the 19mers are generally present within deleted DNA. The working model is that there is a small family of three or four rearrangements, including Tlr1, which contain the 19mer repeat sequences near the rearrangement junctions.

Southern hybridization with Tlr1.C-B (Fig. 1) showed that the innermost half of the inverted repeat belongs to a larger family of repeated, micronuclear-limited sequences with a copy number of about 30 (7). Thus the 19mer repeats of Tlr1 are associated with a larger family of repeated sequences that is also micronucleus-limited.

The objective of this study was to determine whether the *cis*-acting sequences for Tlr1 are contained within the element, or, as has been shown for two of the short elements that undergo developmentally regulated deletion in *Tetrahymena*, in the flanking DNA.

MATERIALS AND METHODS

Cell strains

Strains CU428, Mpr/Mpr (6-mp-s, VII) and CU427, Chx/Chx (cy-s, VI) of inbreeding line B were obtained from P. Bruns (Cornell University).

DNA constructs

All constructs were first built between the two *NotI* sites in the multiple cloning site of the plasmid pHSS6 (14). These clones are designated here as the pH series of constructs. The *NotI* fragments were subsequently excised from pHSS6 and ligated into the *NotI* site of the *Tetrahymena* processing vector pD5H8, producing the constructs designated here as the pD series.

pDWT.cam. Restriction fragments containing the junction sequences of Tlr1 were cloned previously (7). The right end fragment, Tlr.rB-H (Fig. 1), containing 750 of the 825 bp inverted repeat, 48 bp of DNA between the inverted repeat and the major junction site and 51 bp of flanking DNA, was cloned into pHSS6 to create pHMicR. The left end fragment, IIC7, containing 859 bp of mic-limited DNA including the entire inverted repeat and 1039 bp of flanking DNA, was released from pBR322 as an *EcoRI*–*Bam*HI fragment including 346 bp of pBR322 DNA and ligated into pHSS6. To join the left and right end fragments, pHMicR and pHIIC7 were digested with *EcoRI* and *NcoI*. Fragments containing the Tlr1 repeats were gel purified from each digestion and ligated together. The resulting construct, pHIIC7+MicR, had the Tlr1 repeats in an

inverted orientation in pHSS6. This construct was modified to contain the entire 825 bp inverted repeat from the right side and the adjacent *HindIII* fragment, Tlr1.rH-H1. The right end *EcoRI*–*NotI* fragment of pHIIC7+MicR was replaced with a fragment from pBskMicRWT (a gift from D. Wexler) containing the complete inverted repeat, 125 bp of micronuclear DNA internal to the repeat and flanking sequences from the right side extending to the second *HindIII* site, to create pHWT (wild-type).

The presence of repeated sequences in the insert led to non-specific recombination events that deleted the entire insert or a part of it in *Escherichia coli* cells. To allow for selection of transformants with intact construct, a chloramphenicol resistance gene (*cm^R*) was cloned within the inverted repeat sequences. A 3.8 kb *SmaI* fragment containing the *cm^R* gene fragment was released from plasmid pMOB45 (15) and *EcoRI* linkers were added for ligation into the *EcoRI* site of pHWT to create pHWT.cam.

To allow for transformation into mating *Tetrahymena* cells, the *NotI* cassette was excised from pHSS6 and ligated into the *NotI* site of the *Tetrahymena* rDNA shuttle vector pD5H8 (16), producing pDWT.cam.

Construction of pDIR.cam. pHWT.cam was digested with *HindIII* to release three *HindIII* fragments consisting of IIC7+MicR (6.74 kb), the right side flanking sequence (Tlr1.rH-H1, 730 bp) and the pHSS6 vector with pBR322 sequences (2.5 kb). The *HindIII* digestion mixture was purified and religated without separating the individual fragments. A clone which lacked the Tlr1.rH-H1 fragment (pHIR.cam) was identified and the orientation of the *HindIII* fragments was confirmed by restriction mapping. The 7.11 kb IR.cam *NotI* fragment was purified and ligated into the *NotI* site of pD5H8 to create pDIR.cam.

Construction of pDΔ.cam. A clone lacking the 19mer repeats from the right half of the Tlr1 inverted repeat was provided by D. Wexler. This MicRΔ construct contained an internal deletion of the 396 bp IIC7.1b fragment, from the *Clal* to the *RsaI* site of the inverted repeat. The *EcoRI*–*NotI* MicR fragment from pHIIC7+MicR was replaced with MicRΔ to create pHΔ. The *cm^R* gene fragment was added as described above. The 7.44 kb *NotI* Δ.cam insert fragment was ligated into the *NotI* site of pD5H8 to create pDΔ.cam.

Tetrahymena conjugation and transformation

Tetrahymena strains CU427 and CU428 were used in all the mating and transformation experiments. Matings were as described by Bruns and Brussard (17). Mating pairs were fixed by mixing 20 μl of the mating culture with 10 μl Schaudinn's fixative (two parts saturated HgCl₂, one part absolute ethanol) and examined under the microscope for the presence of developing macronuclei. Two hours past the point where 50% of the mating pairs showed anlagen, usually 8–10 h of mating, the mating cells were transformed by electroporation. The cells were electroporated according to the protocol developed by Gaertig and Gorovsky (18). Paromomycin was added to a final concentration of 100 μg/ml 20–24 h post-electroporation. Transformants were selected 2–4 days after adding paromomycin and were subsequently grown in 20 ml 2% PPYS with 100 mg/ml paromomycin for DNA isolation.

Whole cell DNA was isolated from *Tetrahymena* by a modification of the method of Austerberry and Yao (19). Briefly, the cells were pelleted, then lysed with SDS and proteinase K at 65°C. The DNA was precipitated with 12% polyethylene glycol in 1.2 M NaCl and spooled out with a pasteur pipette. It was rinsed in 70% ethanol, dried and redissolved in TE pH 8.0. The sample was treated with RNase, extracted with phenol:chloroform (24:1) and precipitated with 0.5 vol 7.5 M NH₄OAc and 0.54 vol isopropanol. In some cases, the DNA was spun at 13 000 r.p.m. for 45 min in the microfuge to pellet contaminating carbohydrates before precipitation with isopropanol.

Southern analysis

Whole cell DNA was digested with the appropriate restriction enzymes, size fractionated by gel electrophoresis through 0.8–1% agarose and transferred to Genescreen nylon filters (NEN) by the downward capillary method (20). Probes were labeled using the random primer method (21). All blots were probed with a 726 bp *HindIII*–*Sau3A* fragment (IIC7.1a, Fig. 1) that is specific for DNA flanking Tlr1 on the left side.

Polymerase chain reaction (PCR)

A typical PCR reaction had 20 pmol of each primer, 100 ng of genomic template DNA, 2.5 mM MgCl₂, 0.2 mM dNTPs, 10% glycerol, 1× PCR buffer and 0.5 ml *Taq* DNA polymerase (Promega). The primers and DNA template were denatured in the thermocycler at 95°C for 1 min and annealed at 55°C for 1 min before adding *Taq* polymerase on ice, then extended in the first cycle. A typical reaction had 35 cycles of 95°C for 1 min, 55–60°C for 1 min, 72°C for 1 min followed by the last cycle at 72°C for 7 min. The sequences of the primers are provided in Table 1.

Table 1. Primers

Primer ^a	Sequence
Tet 1	5'-AAATGAGAAATTTTAAAAATTTTAGAAACG-3'
Tet 2	5'-ACGTGAGAACTTTAGAACTTGAGAAAAAT-3'
Tet 3	5'-GTAGAATATTTTTTTACCTGTACTGATCT-3'
Tet 4	5'-AAATGCTCCGATGTAAATCTCTCTCG-3'
Tet 5	5'-GCTTACATATAATTATCTGCTTCTTATACGA-3'
Tet 6	5'-ACTATGATTCCTCGTAAGCTTCACTTACA-3'
Tet 7	5'-TATATTTCTTATTTCTTTTATTTTCTCAAG-3'
C1	5'-TACCTACCAGTTCTCCGCCT-3'
C2	5'-GATGCAAGCAGCTGGAAGG-3'
C4	5'-TAGCAATTTAACTGTGATAAACTACCGCA-3'
C4	5'-GATAAGCTGTCAAACATGAGAATTCGG-3'

^aTet, *Tetrahymena* DNA primers; C, construct-specific primers.

DNA sequencing

For sequencing the PCR products directly, the USB sequencing kit from Amersham Life Science was utilized following manufacturer's protocols. Sequencing was typically done with end-labeled primer, in the thermocycler for 35 cycles consisting of 94°C for 1 min followed by 60°C for 1 min, beginning with a preheated thermocycler. Alternatively, direct sequencing was done in the thermocycler using [α -³²P]dATP in the sequencing reaction. Sequenced products were run out

on a 6% denaturing gel for 2–4 h. The gel was dried for 1 h and exposed to X-ray film.

RESULTS

The inverted repeat plus several hundred base pairs of flanking DNA is sufficient for Tlr1 rearrangement

Cis-acting sequences for DNA rearrangement in *Tetrahymena* can be tested *in vivo* by analysis of rearrangement of constructs in the rDNA vector pD5H8 (22). Micronuclear sequences cloned on this vector and introduced into the macronuclear anlagen of mating cells undergo DNA rearrangements similar to chromosomal sequences.

The salient features of the Tlr1 rearrangement are shown in Figure 1. In order to obtain a manageable construct for an *in vivo* assay of Tlr1 DNA rearrangement, a plasmid was built containing the inverted repeat along with 796 bp of DNA flanking the inverted repeat on the left and 830 bp on the right, omitting the 11 kb or more of DNA between the two halves of the inverted repeat. Early versions of the construct were unstable in a variety of recombination deficient *E.coli* strains, presumably due to the presence of the long inverted repeat. In order to overcome this problem, a gene conferring chloramphenicol resistance (*cm^R*) was cloned within the insert. This allowed for selection of bacterial clones containing the intact construct. Since the resistance gene was placed within the deleted DNA, it was not expected to affect DNA rearrangement in *Tetrahymena*.

Mating cells were transformed with the construct by electroporation at 8 h after mixing the two mating types. The timing correlates with DNA rearrangement in the developing macronucleus, which is followed by rDNA amplification. About 30 *Tetrahymena* transformants were selected based on resistance to paromomycin, conferred by a mutation in the 17S rRNA gene of the vector. Whole cell DNA was isolated from six independent lines, digested with *Bam*HI to release the construct from rDNA, and analyzed by Southern hybridization (Fig. 2). The blots were hybridized with a probe specific for the left junction of Tlr1. DNA from a line transformed with vector alone, included as a negative control (lane 8), showed no hybridization. At the exposures used, no hybridization was seen to the chromosomal macronuclear DNA. The construct is present on the rDNA, therefore it is at a 200-fold higher copy number than the chromosomal sequence.

A 2.2 kb band was present in all the transformant lanes, indicating that the construct was rearranged faithfully in all six lines analyzed (Fig. 2). The majority (60–90%) of the DNA was in the 2.2 kb band. The rearranged product was PCR amplified from the *Tetrahymena* transformants using a *Tetrahymena* primer Tet 6 from the macronuclear-retained DNA at the right side and a primer specific to the bacterial plasmid pBR322 region of the construct (C4) (to prevent amplification of chromosomal macronuclear DNA). The expected 1.2 kb product was obtained from all the transformants. No PCR product was obtained from the DNA of untransformed cells. The 1.2 kb PCR product was gel purified and sequenced directly using radiolabeled Tet 6 primer. For two of the transformants, a unique sequence was determined for the junction (Table 2). For four of the transformants, the film showed clean sequence up to a point at which multiple bands appeared. Since

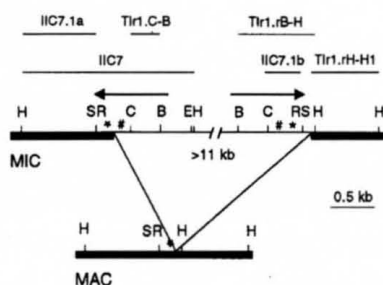


Figure 1. Restriction map of Tlr1. The arrows represent the long inverted repeat. Within the inverted repeat are the 19mer tandem repeats: *, 19A (ATTATTTCTTTTACATT) and #, 19B (TTTCTCATTATGAAAAG). The bold lines represent macronucleus-destined DNA and the thin lines micro-nucleus-limited DNA. The lines above the map indicate the fragments used in cloning and for making probes. B, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; R, *Rsa*I; S, *Sau*3A.

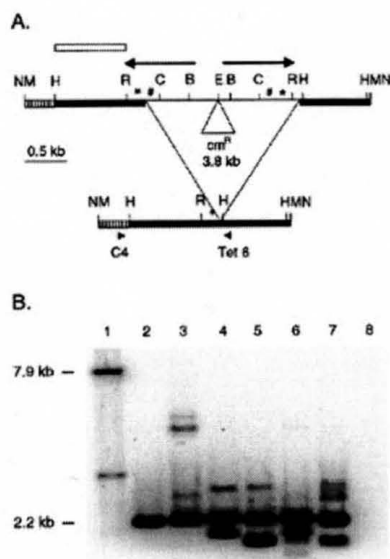


Figure 2. Rearrangement of a construct with the Tlr1 inverted repeat and flanking sequences. (A) The WT.cam construct and the major rearrangement product. Bold lines represent macronuclear DNA and the thin line is the micro-nucleus-limited DNA. The arrows show the inverted repeat. *, 19A tandem repeats; #, 19B tandem repeats; hatched bar, 346 bp of pBR322; *cm*^R, chloramphenicol resistance gene; B, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; M, *Bam*HI; N, *Not*I; R, *Rsa*I. The *Not*I or *Bam*HI fragment of the unrearranged construct is expected to be 2.2 kb. (B) Southern analysis of pDWT.cam transformants. Whole cell DNA was digested with *Bam*HI and probed with the probe indicated by the open bar in (A). Lane 1 contains pDWT.cam plasmid DNA digested with *Bam*HI to release the 7.9 kb unrearranged construct fragment. Lanes 2–7 have DNA from pDWT.cam transformants C10, F5, A8, H3, F2 and G3 that show a 2.2 kb band corresponding to accurately rearranged construct. Lane 8 contains DNA from a cell line transformed with pD5H8 vector.

the sequence of the Tlr1 rearrangement is known and much of the microheterogeneity is within a track of A residues, it was possible to follow the sequences of two or even three rearranged products within a single transformant. The

sequence analysis revealed that the rearranged products showed junctional diversity or microheterogeneity over a range of 8–10 bp, similar to that for the chromosomal Tlr1 rearrangement (13).

Table 2. Sequence of the Tlr1 WT.cam construct rearrangement junctions

Transformant	Sequence ^a
F5	CTAA <u>AAAA</u> agatt
A8	CTAAAGTTaaaagatt
	CTAAAGTaaaagatt
B3	CTAAAGTTaaaagatt
	CTAAAGTTaaaagatt
B6	CTAAAGTaaaagatt
G3	CTAAAGTaaagatt
	CTAAAGTaaaagatt
	CTAAAGTaaaagatt
C10	CTAAAGTTCTCaagatt

^aUpper case represents sequence from the left side and lower case from the right side. Underlined sequence may be from either side. PCR products were purified and sequenced directly.

In addition to the band representing the major rearrangement, there were also variant bands. The sizes of some of the fragments corresponded to the naturally occurring variants previously seen in the deletion of the chromosomal Tlr1 element (7). Other minor bands detected in the transformants that did not correspond to the chromosomal Tlr1 rearrangement are likely to be artefactual, since some aberrant rearrangement is not uncommon in this system (9).

No unrearranged DNA was detected even after long exposures. Thus, rearrangement of the construct mimicked rearrangement of the chromosomal element in efficiency, junction variability and junction microheterogeneity. The experiment demonstrated that the entire inverted repeat and several hundred base pairs of flanking DNA are sufficient for an accurate DNA rearrangement of Tlr1 *in vivo*. The internal 11 kb or more of micronuclear-limited DNA is not required.

Flanking DNA is required for accurate Tlr1 rearrangement

To determine whether the flanking region of Tlr1 contained *cis*-acting signals for DNA rearrangement, a construct was built that lacked some of the flanking sequences. The left end of Tlr1 was not a suitable target for this experiment because deletion of the left flanking sequences would involve deletion of a substantial portion of the inverted repeat and thus not permit distinction of the roles of the flanking sequences and the inverted repeat. The right flanking sequences were chosen because the right half of the inverted repeat is entirely deleted in the major rearrangement and all the naturally occurring variants. The construct pDIR.cam was similar to the first construct, with the difference that the Tlr1.rH-H1 fragment was removed, leaving only 51 bp of mac destined sequences to the right of the major rearrangement junction site.

Mating *Tetrahymena* cells undergoing DNA rearrangement were transformed with pDIR.cam by electroporation. Twelve paromomycin resistant *Tetrahymena* transformants were obtained. Whole cell DNA was isolated from nine of these, digested with *Not*I to release the insert and analyzed on

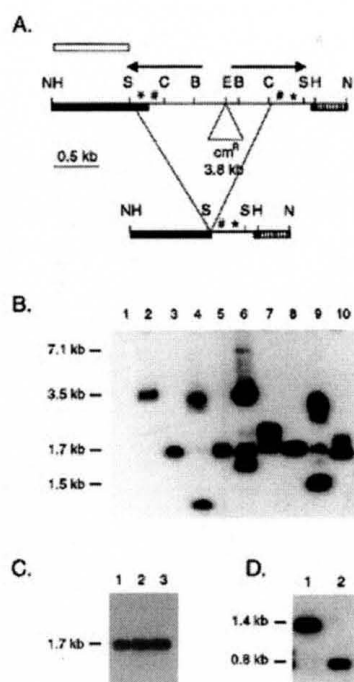


Figure 3. Rearrangement of a construct without the *Hind*III fragment containing flanking sequences to the right of the element. (A) Restriction map of the IR.cam construct and the predominant rearrangement product, *ap1.7*. The bold lines represent macronuclear DNA and the thin line micronuclear-limited DNA for the major chromosomal rearrangement. Arrows, inverted repeat; *, 19A tandem repeats; #, 19B tandem repeats; hatched bar, 346 bp of pBR322; *cm^R*, chloramphenicol resistance gene; B, *Bgl*III; C, *Cla*I; E, *Eco*RI; H, *Hind*III; N, *Not*I; S, *Sau*3A. The open bar indicates the probe for the blots in (B), (C) and (D). The *Not*I fragment of the unrearranged construct is 7.1 kb and the rearranged product utilizing the junctions used *in vivo* is expected to be 1.5 kb. (B) Southern analysis of pDIR.cam transformants. Whole cell DNA was digested with *Not*I to release the construct from the rDNA. Lane 1, negative control lane containing DNA from a pD5H8 transformant. Lanes 2–10, DNA from independent pDIR.cam transformants. Seven of the nine transformants show an aberrant rearrangement that produced a 1.7 kb *Not*I fragment. (C) Whole cell DNA from pDIR.cam transformant A8 digested with *Not*I in lane 1, *Not*I and *Cla*I in lane 2, and *Not*I and *Bgl*III in lane 3. (D) Whole cell IR.cam transformant A8 DNA digested with *Hind*III in lane 1, and *Hind*III and *Sau*3A in lane 2.

Southern blots probed with sequences specific to the left of the Tlr1 element (Fig. 3A).

If flanking sequences are not essential for Tlr1 rearrangement, micronucleus-limited sequences would be deleted from the 7.11 kb Tlr1 *Not*I fragment of pDIR.cam to generate a 1.5 kb *Not*I fragment. If sequences in Tlr1r.H-H1 are required for Tlr1 rearrangement, the Tlr1 sequences in the construct were expected to remain unrearranged or undergo aberrant rearrangement. The pDIR.cam construct underwent aberrant DNA rearrangement in all the transformant cell lines (Fig. 3B). Multiple bands ranging from 0.75 to 3.1 kb were detected. A 1.5 kb *Not*I fragment was seen in only one of nine transformant lines examined.

A 1.7 kb band was detected in seven of nine pDIR.cam transformants. The presence of a common rearrangement product in

multiple transformants suggests that, in the absence of the wild-type *cis*-acting signal, a cryptic signal may have been utilized to determine the junctions. PCR amplification of this product was attempted several times under varying conditions without success. Aberrant amplification was observed which may have been due to the presence of hairpin structures during annealing. Therefore, the junctions of the 1.7 kb aberrant product (*ap1.7*) were mapped using Southern and PCR analyses.

Southern analysis was performed on a transformant that contained only *ap1.7* and no other rearrangement variants (Fig. 3B, lane 8). To map the left boundary, whole cell DNA was digested with *Not*I alone or *Not*I together with *Cla*I or *Bgl*III. A 1.7 kb fragment was detected in all three lanes (Fig. 3C). This showed that both of the *Cla*I sites, together with the intervening DNA, were deleted in the rearrangement to *ap1.7*.

In another Southern experiment, whole cell DNA was digested with *Hind*III or *Hind*III and *Sau*3A (Fig. 3D). An ~1.4 kb fragment was observed with the *Hind*III digestion and a 0.8 kb fragment was seen in the *Hind*III and *Sau*3A double digest, suggesting that *ap1.7* retained the *Hind*III and the *Sau*3A sites from the left side. Thus, the left junction of *ap1.7* lies between the *Sau*3A and the *Cla*I sites (Fig. 3A).

A PCR experiment was performed to map the junctions of *ap1.7* more closely (Fig. 4). A series of oligonucleotides from within the inverted repeat were used against construct-specific oligonucleotides from both sides to determine which micronuclear sequences were retained in *ap1.7*. As a positive control, the inverted repeat primers were used against a *Tetrahymena* DNA primer Tet 5 (Fig. 4B, lanes 1, 4 and 7). All the products expected for amplification of the chromosomal DNA were obtained from the control reactions (159 bp with Tet 4, 191 bp with Tet 3 and 388 bp with Tet 2), indicating that the primers and the template DNA were compatible under these PCR conditions.

To map the left boundary of *ap1.7*, two construct-specific primers (C1 and C2) were used with the same set of Tlr1 primers (Tet 2, Tet 3 and Tet 4). PCR products of 921 and 861 bp were obtained with the oligonucleotide Tet 4 and construct-specific primers C1 and C2, respectively. No products were obtained with oligonucleotides Tet 3 or Tet 2 (Fig. 4B). Since Tet 3 did not produce a PCR product with the construct-specific primer, some part of the sequence corresponding to Tet 3 primer is deleted from the rearranged aberrant product. This suggested that the left boundary of *ap1.7* is within 27 bp of the *Sau*3A site.

Southern analysis indicated the junction of the *ap1.7* rearrangement is within a 1.4 kb *Hind*III fragment. The PCR analysis of the left boundary suggests that only ~854 bp of that can be derived from the left side (826 bp of IIC7.1a + 27 bp or less beyond the *Sau*3A site). Thus, ~550 bp of DNA inside the right *Hind*III site of the construct must be retained in *ap1.7*. This was supported by PCR analysis of the right boundary of the rearrangement. A set of Tlr1 primers (Tet 1, Tet 2 and Tet 3) were used against two construct-specific primers C3 and C4. Negative control experiments verified that these primer pairs did not amplify the macronuclear chromosomal DNA of a non-transformed cell line. PCR products were obtained with all three primer sets from within the inverted repeat, suggesting that the sequence corresponding to those primers was retained

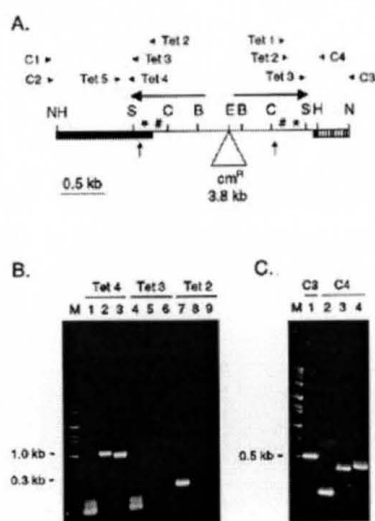


Figure 4. PCR mapping of *ap1.7*. (A) Diagrammatic representation of the pDIR.cam construct showing the location of the oligonucleotide primers. Vertical arrows indicate the boundaries of the deleted region for *ap1.7* (B and C) PCR amplification of whole cell DNA from the IR.cam A8 transformant. M, marker lanes. (B) Left end: lanes 1–3 were primed with Tet 4, lanes 4–6 with Tet 3 and lanes 7–9 with Tet 2. The first of each set was against *Tetrahymena* DNA specific oligonucleotide Tet 5; the second and third sets with construct-specific oligonucleotides C1 and C2, respectively. (C) Right end: lane 1 was primed with Tet 3 and construct-specific oligonucleotide C3. Lanes 2, 3 and 4 were primed with Tet 3, Tet 2 and Tet 1, respectively, against construct-specific oligonucleotide C4.

in *ap1.7* (Fig. 4C). Their sizes (539 bp with Tet 3 + C3, 188 bp with Tet 3 + C4, 385 bp with Tet 2 + C4 and 414 bp with Tet 1 + C4) agreed with the Southern data. Thus, the right junction of *ap1.7* can be narrowed to 175 bp between the *ClaI* site and the Tet 1 primer sequence (Fig. 4A), and is likely to be within 20–30 bp of the *ClaI* site.

To verify that the right boundary of the rearrangement in the other transformants was within the inverted repeat, a PCR was performed on DNA from two additional transformants which showed only the 1.7 kb *NotI* band on the Southern blot (Fig. 3, lanes 3 and 5). Using oligonucleotides C4 and Tet 3 as primers, the expected PCR product of 188 bp was obtained from both, suggesting that the right junction was within the inverted repeat in these two transformants as well (data not shown).

In conclusion, Southern hybridization and PCR data show that the left boundary of the predominant aberrant rearrangement, *ap1.7*, is ~32–60 bp within the inverted repeat, close to the boundary of the naturally occurring variant *rv0.9* (13). The aberrant characteristic of *ap1.7* is the right boundary, which is within the inverted repeat, unlike any Tlr1 chromosomal rearrangement seen to date. The inaccurate rearrangement of pDIR.cam suggests that the 0.7 kb *HindIII* right side flanking DNA fragment contains *cis*-acting sequences that are required for determination of the junction site.

19mer sequences are not required on both sides of the construct

The most striking structural feature of Tlr1 is the inverted repeat sequence. The inverted repeat contains tandem repeats

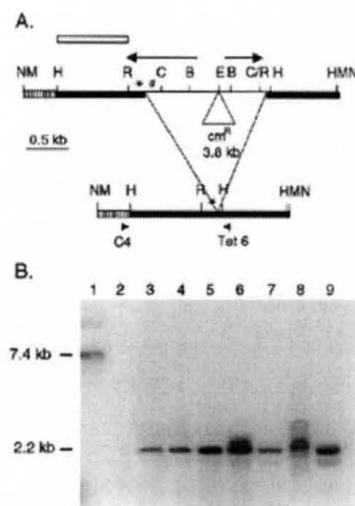


Figure 5. Rearrangement of a construct with deletion of the 19mer repeats from the right inverted repeat. (A) Restriction map of the pDA.cam construct and the rearrangement. The bold lines represent macronuclear DNA and the thin line micronuclear-limited DNA. Arrows, inverted repeat; *, 19A tandem repeats; #, 19B tandem repeats; hatched bar, 346 bp of pBR322; cm^R, chloramphenicol resistance gene; B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; M, *Bam*HI; N, *Not*I; R, *Rsa*I. The open bar indicates the probe for the blot in (B). The unrearranged construct is within a 7.4 kb *Bam*HI fragment and for the accurately rearranged product the fragment is expected to be 2.2 kb. (B) Southern blot of whole cell DNA digested with *Bam*HI to release the 7.44 kb unrearranged construct. Lane 1 contains the pDA.cam plasmid DNA restricted with *Bam*HI to release the 7.44 kb unrearranged construct. Lane 2 contains DNA from a pD5H8 transformant as a negative control. Lanes 3–9 contain DNA from pDA.cam transformants, and show a 2.2 kb band corresponding to an accurately rearranged construct.

of two different 19mer sequences, 19A and 19B. Southern analysis indicates that the 19mer repeats are conserved amongst other putative Tlr1 family members (7). The next objective was to determine whether the 19mer tandem repeat sequences were required for Tlr1 rearrangement. The 19mer sequences cannot be removed from the left side of the construct because that region contains the rearrangement junction. The 19mer region (fragment IIC7.1b) was removed from the right side of the inverted repeat. The resulting construct, pDA.cam, was identical to the control construct with the exception that the 393 bp *ClaI*–*RsaI* fragment, IIC7.1b (Fig. 1), was deleted from the right half of the inverted repeat. This left 432 bp of the 825 bp inverted repeat in the construct, including the terminal 33 bp and 399 bp internal to IIC7.1b.

The pDA.cam construct was introduced into mating cells by electroporation and 42 paromomycin resistant *Tetrahymena* transformants were obtained. Whole cell DNA was isolated from seven transformants, digested with *NotI* to release the construct from the rDNA and analyzed by Southern hybridizations (Fig. 5). In all seven transformants there was a 2.2 kb *NotI* fragment, the size expected for accurate deletion at the junctions of the major chromosomal rearrangement.

In order to verify the location of the rearrangement junction, the rearranged product was PCR amplified from the transformants. Whole cell genomic DNA was amplified using one primer

specific to the construct, C4, and the other primer specific to *Tetrahymena* DNA, Tet 6 (Fig. 5). This prevented amplification of the chromosomal macronuclear DNA. The expected 1.2 kb product was obtained from all the transformants (data not shown). No PCR product was seen from a non-transformed line, confirming the construct specificity of the primers.

Thus, the Southern and PCR results concur and show that the pDA.cam construct was rearranged accurately, similar to the Tlr1 chromosomal rearrangement. This shows that the 19mers are not required on both sides of the Tlr1 construct for correct rearrangement.

DISCUSSION

A series of plasmid constructs containing the Tlr1 element were built and assayed for DNA rearrangement *in vivo*, in order to identify sequences required for DNA rearrangement. The first construct showed that the entire inverted repeat, together with 796 bp of flanking DNA on the left and 831 bp on the right is sufficient for accurate rearrangement. The internal 11 kb or more of the element is not required. This is comparable to the correct rearrangement of the M element construct that lacked 395 of the 600 bp M element (9). The rearranged WT.cam construct products showed junction variability and microheterogeneity similar to those in the macronuclear Tlr1 products of the chromosomal rearrangement.

The significance of the flanking region in Tlr1 rearrangement was revealed by aberrant rearrangement of the second construct, pDIR.cam, which had only 51 bp of sequence flanking the major Tlr1 junction. Instead of the 1.5 kb *NotI* fragment expected for joining as in the major chromosomal rearrangement of Tlr1, the *NotI* fragments containing the rearrangement junction ranged from 0.75 to 3 kb. Analysis of the most common rearrangement product (*apl.7*) obtained from pDIR.cam revealed that the left junction was close to that found in the minor Tlr1 variant rv0.9 (13). The right junction, however, was ~400 bp inside of the right inverted repeat. The right junction is outside of the inverted repeat in all the Tlr1 chromosomal rearrangements analyzed to date. The fact that the right joining site of *apl.7* was so far inside the inverted repeat suggests the right flanking sequence contains important *cis*-acting sequences that designate the right boundary of the rearrangement. This is reminiscent of the M rearrangement in which short repeats in the flanking DNA determine the rearrangement junction. No well-conserved motif for Tlr1 or its cryptic partner was identified despite extensive scrutiny of the flanking Tlr1 sequence.

Another construct was built to test the role of the 19mer tandem repeats located within the Tlr1 inverted repeats, which are conserved at other sites in micronuclear-limited DNA. In the pDA.cam construct, the 19mer sequences were deleted from one of the inverted repeats. Surprisingly, pDA.cam underwent rearrangement and utilized the same junctions as the major Tlr1 rearrangement product. Thus, removal of the 19mer sequences from one side did not affect DNA rearrangement. There are several possible explanations for this result. First, the 19 bp repeats may be required at only one end of the element. That would be consistent with the postulated redundancy of rearrangement promoting sequences within the M element (23). In principle, it would be interesting to delete the 19mer region from both ends of the construct. However, since the

major chromosomal junction for the Tlr1 rearrangement is within this region (Fig. 1), the results of that experiment would be difficult to interpret.

Second, the 19mer sequences may be required for rearrangement on the chromosome but not on the construct. For example, the inverted repeats may be involved in bringing the macronuclear junctions of this large element in close proximity to allow the rearrangement reaction to occur. In the construct there is less internal DNA and the junctions are closer together than they are on the chromosome, therefore the repeat sequences may not be required.

Third, the inverted repeats may be involved in some process other than developmentally regulated DNA rearrangement, such as transposition within the micronuclear genome. We do not favor this hypothesis because the 19mer region binds developmentally regulated proteins that are detected only at the time of DNA rearrangement (Ellingson and Karrer, unpublished data).

The Tlr1 element with its long inverted repeat bears a structural resemblance to transposable elements in *Drosophila* (24), sea urchins (25) and the hypotrichous ciliates (26,27). Critical sequences for the transposition of the elements in sea urchins and the ciliates have not yet been identified. The subterminal region of the P element of *Drosophila* binds the transposase protein and is required for P element transposition (28,29). Although the 19mer sequences of Tlr1 bind developmental stage specific proteins *in vitro*, this region is not required at both ends of the element for Tlr1 rearrangement.

This study revealed that *cis*-acting sequences defining the junction site of Tlr1 are present outside of the deleted DNA. Transposable elements, on the other hand, are self-contained in their sequence requirements and have no dependence on flanking DNA. Thus, though Tlr1 structurally resembles transposable elements, it is similar to other micronuclear-limited elements in *Tetrahymena* with respect to its *cis*-acting sequence requirements.

ACKNOWLEDGEMENTS

We thank Michael Cox and Sergei Saveliev, in whose laboratory some of the sequencing work was done, for providing reagents, guidance and support. We also thank Daniel Wexler for help in building some of the DNA constructs. This work was supported by grant MCB-9631404 from the National Science Foundation. N.S.P. was supported in part by an Arthur J. Schmitt fellowship and by a Marquette University fellowship.

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