studies will be needed to clarify the relationship between the hippocampus and the premotor and prefrontal cortices in associative memory formation. We found that although both sustained and baseline-sustained changing cells signaled when learning occurred, only the sustained changing cells continue to signal selective information after learning. We hypothesize that these sustained changing cells not only participate in the formation of associative memories but also may participate in the neural circuit important for the eventual storage of these associations in long-term memory.

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25. Supported by grants from the National Institute of Mental Health and a joint Bioengineering Research Partnership grant from the National Institute on Drug Abuse (E.N.B. and W.A.S.) and grants from the McKnight Foundation and the John Merck Fund (W.A.S.). We thank C. Chiu and N. Nystrom for help with the recording experiments; S. Fujisawa, A. Schain, and T. Shah for early contributions to the analysis; and T. Movshon for helpful comments on the manuscript.

Supporting Online Material

www.sciencemag.org/cgi/content/full/300/5625/1578/DC1
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10 March 2003; accepted 7 May 2003

Programmed DNA Deletion As an RNA-Guided System of Genome Defense

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Genomewide DNA rearrangements occur in many eukaryotes during development, but their functions and mechanisms are poorly understood. Previous studies have implicated a sequence-recognition mechanism based on RNA-mediated interactions between nuclei in ciliated protozoa. In this study, we found that the process recognized and deleted a foreign gene integrated in a *Tetrahymena* chromosome, suggesting an unusual mechanism of genome surveillance. We further found that injection of double-stranded RNA into the cell at specific developmental stages triggers efficient deletion of the targeted genomic regions. Together the results indicate an RNA-based mechanism that directs genomewide DNA rearrangements and serves to disable invading genetic agents.

The ciliated protozoan *Tetrahymena thermophila* contains a germinal nucleus (micronucleus) and a somatic nucleus (macronucleus) in each cell. During sexual conjugation, the micronucleus goes through a series of events to produce a zygotic nucleus, which divides and differentiates to form the new macro- and micronucleus of the progeny cell. The old macronucleus is destroyed. Formation of the new macronucleus involves extensive genomewide DNA rearrangements. Thousands of specific DNA segments, comprising ~15% of the genome, are deleted, and the remaining DNA is fragmented and endoduplicated about 23-fold to form the somatic genome, which is responsible for all transcriptional activities during growth. These deleted segments (referred to here as deletion elements) range from several hundred base pairs (bp) to more than 20 kb in size and are composed of single-copy and moderately repetitive sequences. Some of them are deleted with precise boundaries, whereas for others the boundaries are somewhat variable (*1*). The mechanism, biological role, and evolutionary origin of this deletion process remain largely unknown.

Although programmed deletion of micronucleus-specific sequences is a tightly regulated process, several notable exceptions have been observed to occur in *Tetrahymena* (*2*) and a related ciliate, *Paramecium* (*3*, *4*). Through genetic manipulations, a cell can be modified to contain a particular micronucleus-specific sequence in the macronucleus. This produces an unusual phenomenon: In subsequent matings of this cell, the presence of the anomalous sequence in the old macronucleus prevents this sequence from being deleted in the newly formed macronucleus. These data indicate that, through some type of internuclear communication, a ciliate cell can distinguish sequences that are present only in the germ line from those also present in the somatic genome. Through this same process, the cell could potentially identify the sequences destined for deletion. It suggests a possible mechanism for deleting micronucleus-specific sequences without the need for a specific sequence signal. If true, foreign sequences that are inserted only into the germline genome will also be deleted in the daughter macronucleus through this process.

To test this idea directly, we inserted a bacterial sequence into the germline genome of *Tetrahymena* with the use of homology-directed gene replacement (*5*). The inserted sequence is a 1.5-kb knockout cassette containing the neomycin-resistant gene (*neo*) of *Escherichia coli* flanked by *Tetrahymena* regulatory sequences (*6*). We inserted this sequence downstream of the single ribosomal RNA gene, replacing a 4.2-kb region that includes a normal deletion element with variable boundaries (*7*, *8*) (Fig. 1A). Strains of compatible mating types were generated that were homozygous for this cassette in their micronuclei but lacked the transgene in their macronuclei (*5*). They were crossed to one another to produce progeny for DNA analysis. The macronucleus of the progeny was expected to contain the transgene because it is descended from the parental micronuclei. However, smaller DNA fragments were detected (Fig. 1B) that apparently lost the transgene cassette through simple deletions (Fig. 1A). The deleted regions are slightly heterogeneous, with their right deletion boundaries falling just inside the cassette and the left boundaries just outside of it (fig. S1). We tested the same transgene at two additional

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loci that unlike the rDNA locus are not normally involved in DNA deletion. At the condensin *tSMC-4* locus (*9*), a similar approach yielded the same results: The transgene cassette was deleted with heterogeneous boundaries (Fig. 2A). For the linker histone *HH1* locus (*10*), we mated heterozygote strains with a wild-type test strain, and deletion still occurred in many progeny, although at lower overall efficiencies (Fig. 2, B and C). One parental strain (G0-A) contained some transgene copies in the macronucleus. These copies have little effect on deletion, which agrees with earlier results that a certain number of copies are needed for epigenetic inhibition of deletion (*2*, *3*, *11*). In this locus, a region smaller than the 1.5-kb cassette was deleted. The deletion boundaries are closer to the outer ends of the *E. coli* sequence than to the edges of the cassette (Fig. 2B and fig. S1). These boundaries suggest that the deletion process can distinguish between foreign sequences (the *neo* gene) and ectopic *Tetrahymena* regulatory sequences in the cassette.

These results show that a foreign sequence inserted in the germline genome is selectively deleted during new macronuclear development and suggest that a specific DNA sequence element is not necessary for DNA deletion. Whatever its mechanism, the process is an effective method of removing invading foreign sequences from the somatic genome. In most models of genome surveillance, foreign sequences such as transposons are recognized through sequence duplications (*12*–*14*). However, little is known about the recognition of single-copy foreign sequences in eukaryotes.

A key to understanding programmed DNA deletion in *Tetrahymena* is identifying the messenger responsible for the communication between the old macronucleus and the developing macronucleus. RNA is an ideal candidate molecule for this role (*3*), and its involvement has been implicated in earlier studies. Micronuclear elements destined for deletion in the macronucleus are transcribed from both strands specifically during conjugation (*15*). Furthermore, ~28-nucleotide RNAs were recently found in *Tetrahymena* during conjugation, implicating the involvement of an RNA interference (RNAi)–related process in deletion (*16*). Lastly, DNA rearrangements are disrupted by mutations in *TWI1* and *PDD1*, two genes that are related to genes involved in RNAi in other organisms (*16*, *17*). Taking these results together, we speculated that RNAs produced from micronuclear sequences could serve as the primary signal to induce elimination of the transcribed sequences during development.

We tested this idea directly by using RNA to induce deletion of normally retained sequences, because specific sequence signals are not required for deletion. RNA from both strands of sequences that are normally present in the macronucleus was prepared by in vitro transcription and injected into the cytoplasm of mating pairs during conjugation (*5*). Three regions were independently tested: the coding regions of *HH1* (584 bp) and the gene *LIA1* (896 bp) (*18*) together with the upstream region of the α-tubulin gene *ATU* (511 bp) (*19*). The injected mating pairs were grown and analyzed for deletions at the targeted regions (*5*). Deletion was found to occur at all three loci. The frequency of injected pairs showing deletion varies depending on the time of injection and is highest (>50%) for injections done at an early stage of new macronuclear development (7.5 to 8.5 hours after mating begins) (*20*). In an affected pair, deletion typically occurs to a large fraction of the chromosome copies (Fig. 3C). The deleted regions have heterogeneous boundaries that often extend well beyond the boundaries of the injected sequences (Fig. 3, A and B). Some deletions are as large as 2.5 kb. They extend beyond the primer sites used for PCR detection but were detectable by Southern hybridization (Fig. 3C).

These results provide direct proof that double-stranded RNA (dsRNA) triggers programmed DNA deletion in *Tetrahymena*, which expands our understanding of the biological effects of RNAi in eukaryotes (*14*). The process of DNA deletion in *Tetrahymena* shares important features with other RNAi-directed processes, such as heterochromatin formation in *Schizosaccharomyces* and *Arabidopsis* (*21*–*23*) and RNA-dependent DNA methylation in plants (*24*–*26*). As in these processes, it involves dsRNA and small interfering RNA and is accompanied by chromatin modifications, including histone acetylation and methylation and accumulation of chromodomain proteins (*17*, *27*, *28*). In *Tet-*

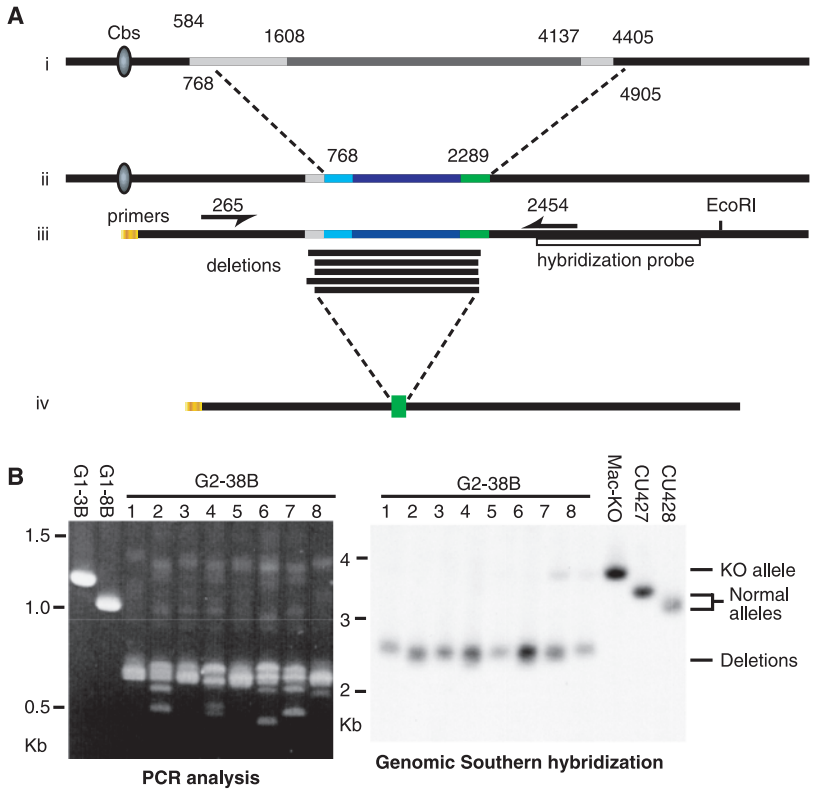

Fig. 1. Deletion of a transgene at the rDNA locus. (**A**) describes this genomic region and the deletion process, with the numbers indicating the relative nucleotide positions in each map: (i) the normal micronuclear locus containing the rdn deletion element (gray) with variable boundaries (light gray) and the chromosome breakage site (Cbs, oval); (ii) the germline knockout chromosome containing *neo* (blue, ~800 bp), 5′ flank of *H4-I* gene (teal, ~330 bp), and 3′ flank of *BTU2* gene (green, ~300 bp); (iii) the expected macronuclear structure if there is no deletion, with new telomere (yellow); and (iv) the actual progeny macronuclear DNA, showing transgene deletion. The black lines represent the extent of individual deletions. The positions of their boundaries (and boundaries of other deletions shown in Figs. 2 and 3) are shown in fig. S1. (**B**) shows PCR and genomic hybridization analysis of transgene deletion. Parental lines G1-3B and G1-8B, containing the transgene only in their micronuclei, were mated to produce progeny pairs G2-38B1 to G2-38B8. CU427 and CU428 are standard laboratory strains that were used for controls. The difference in their fragment sizes is because of natural variations in rdn element deletion. The Mac-KO (KO, knockout) strain contains the transgene only in the macronucleus and is shown for size comparisons. Whole-cell DNA, of which ~95% is macronuclear DNA, was used in this and other experiments and was digested with EcoRI for this hybridization analysis.

rahymena, the marked chromatin is subsequently deleted, but the molecular details of this step are not clear. It could involve some nonspecific nucleases and/or recombinases or even a complex like the RNA-induced silencing complex (RISC) (*14*) containing deoxyribonuclease instead of ribonuclease activities.

Together our results offer an explanation for the control of sequence specificity in genomewide DNA rearrangements in ciliates and suggest a role for DNA deletion in genome surveillance. It appears that, during conjugation, the germline sequences are transcribed to produce dsRNA, which guides specific DNA deletion later in developing macronuclei as proposed previously (*15*, *16*). Thus, dsRNA transcription represents the initial step in providing sequence specificity. Such transcription has been detected from regions surrounding normal deletion elements at early stages of conjugation (3 to 7 hours) (*15*). Whether it also occurs at other genomic regions is unclear. Unregulated transcription is known to occur from *E. coli* sequences inserted in yeast (*29*) and could also occur from the transgene studied here. The old macronucleus provides the second point of control, serving as a gatekeeper to prevent dsRNA with homologous sequences (in the old macronucleus) from initiating deletion in the new macronucleus. The mechanism of this process is unknown but may also involve an RNAi-like process to carry out this sequence-specific interaction. Thus, only micronucleus-specific sequences are deleted. New foreign sequences, such as transposons, that invade the germ line are also deleted, because the occasional transposons that may also insert into the polyploid macronucleus can easily be lost from this nucleus by amitotic division during growth. This process allows transposons to accumulate gradually in the micronucleus and suggests an evolutionary origin for most micronucleus-specific sequences.

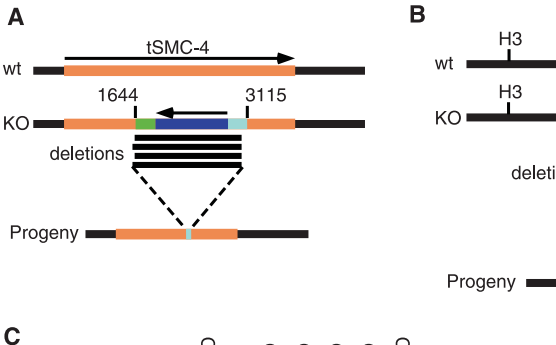

Fig. 2. Deletion of the transgene at the *HH1* and *tSMC-4* loci. Transgene deletions at condensin *tSMC-4* (**A**) and histone *HH1* (**B**) loci are shown. The transgene is shown as in Fig. 1. The coding sequence of each gene is shown in orange, with arrows indicating the direction of transcription. Every deleted region shown was derived from a different pair except the two marked by asterisks, which are from the same pair. wt, wild type. (**C**) shows a Southern hybridization analysis of *HH1* deletions after HindIII digestion. G0-A and G0-C are independent transformed lines that are heterozygous for the transgene in the micronucleus. G0-A mating with CU427 generated the progeny pairs G1-4, G1-5, and G1-8, whereas G0-C mating with CU427 generated G1-11 to G1-14.

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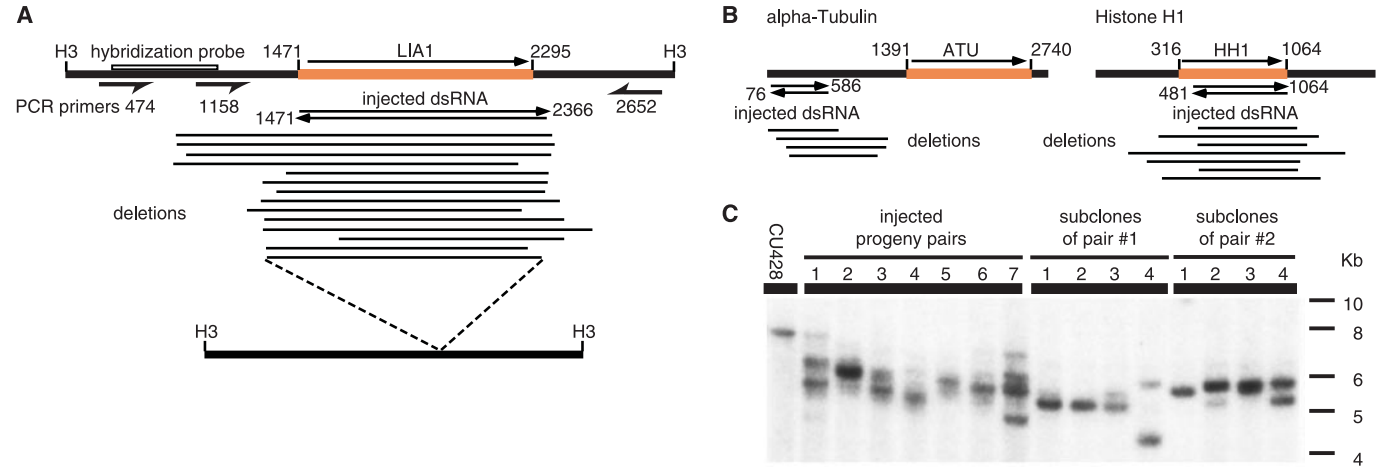

Fig. 3. Injection of dsRNA triggers DNA deletion. (**A**) shows the *LIA1* locus with the locations of the injected dsRNA and the PCR primers indicated on the map. The deleted regions are shown below, along with the macronuclear region after deletion. The top four deletions were cloned with the use of primers 474 and 2652, and the rest were cloned with the use of primers 1158 and 2652. (**B**) shows deletions in the 5′ flank of *ATU* and the coding region of histone gene *HH1* resulting from dsRNA injections. (**C**) shows a genomic hybridization analysis of deletions at the *LIA1* locus. DNA from affected progeny pairs and their subclones, injected at 6 to 7 hours in mating, was digested with HindIII for this analysis.

20. For the LIA1 locus, deletion occurred at the following frequencies after injection of dsRNA: 0 in 34 at 3.5 hours, 1 in 50 at 4.5 hours, 9 in 257 at 6 hours, 11 in 20 at 7.5 hours, and 5 in 18 at 10 hours after mixing cells for mating. Deletion was not detected after injection of single-stranded RNA at similar concentrations at the 6-hour stage (0 in 115 for the sense strand and 0 in 108 for the antisense strand).
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Supporting Online Material
www.sciencemag.org/cgi/content/full/300/5625/1581/DC1
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20 March 2003; accepted 18 April 2003

Nod1 Detects a Unique Muropeptide from Gram-Negative Bacterial Peptidoglycan

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Although the role of Toll-like receptors in extracellular bacterial sensing has been investigated intensively, intracellular detection of bacteria through Nod molecules remains largely uncharacterized. Here, we show that human Nod1 specifically detects a unique diaminopimelate-containing *N*-acetylglucosamine–*N*-acetylmuramic acid (GlcNAc-MurNAc) tripeptide motif found in Gram-negative bacterial peptidoglycan, resulting in activation of the transcription factor NF-κB pathway. Moreover, we show that in epithelial cells (which represent the first line of defense against invasive pathogens), Nod1 is indispensable for intracellular Gram-negative bacterial sensing.

Innate immunity to bacterial pathogens relies on the specific sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition molecules. In mammals, Toll-like receptors (TLRs) represent the most extensively studied class of pattern recognition molecules, which have been shown to sense various PAMPs such as lipopolysaccharide (LPS), peptidoglycan, lipoproteins, double-stranded RNA, and CpG DNA (*1, 2*). Although TLRs are mainly expressed at the plasma membrane, it has been proposed that the Nod molecules—a family of intracellular proteins including Nod1/CARD4 and Nod2/CARD15—could represent a new group of pattern recognition molecules that sense bacterial products within the cytoplasmic compartment, thus allowing detection of intracellular invasive bacteria (*3–9*). We recently showed that Nod1 senses the presence of the Gram-negative pathogen *Shigella flexneri* within the cytoplasmic compartment of epithelial cells (*6*), and we hypothesized that the PAMP detected was LPS, because commercial preparations of LPS were shown to activate Nod1 (*5*). However, because LPS often contains bacterial cell wall contaminants, we investigated in more detail the identity of the molecular motif that is actually detected by Nod1.

Addition of a commercial preparation of *Escherichia coli* LPS (10 μg) to Nod1-overexpressing human embryonic kidney (HEK) 293 cells potentiated the level of Nod1-dependent NF-κB activation by a factor of ~5 (Fig. 1A). By contrast, highly purified *E. coli* LPS (10 μg) or lipid A (10 μg) did not stimulate the Nod1 pathway (Fig. 1A), although they activated macrophages (*10*). We aimed to identify the nature of this LPS contaminant responsible for Nod1 activation. Lipoproteins have been identified as the major contaminants of LPS preparations responsible for TLR2 signaling after stimulation with commercial *E. coli* LPS (*11*). We were unable to stimulate the Nod1 pathway by addition of either synthetic lipopeptide or Lpp, the most abundant lipoprotein in *E. coli* (Fig. 1B). Moreover, boiling or proteinase K treatment of the commercial LPS was not sufficient to abolish Nod1 signaling (*10*).

Peptidoglycans from *E. coli*, *S. flexneri*, *Neisseria meningitidis*, *Bacillus subtilis*, and *Staphylococcus aureus* were purified according to experimental procedures specifically designed for Gram-positive or Gram-negative bacteria (*12, 13*). The harsh purification steps used to purify these peptidoglycans eliminate possible contaminants (fig. S1). Strikingly, we observed that peptidoglycan preparations from Gram-negative bacteria could stimulate the Nod1 pathway, whereas the two Gram-positive peptidoglycan preparations tested here could not (Fig. 1C). Moreover, by using a mutant form of Nod1 that lacks the C-terminal leucine-rich repeats, we observed that Nod1 leucine-rich repeats play a critical role in the sensing of Gram-negative peptidoglycan (fig. S2). Therefore, these results strongly suggest that Nod1 is an intracellular pattern recognition molecule that specifically senses Gram-negative peptidoglycan through the leucine-rich repeat domain.

To identify the peptidoglycan motif detected by Nod1, we analyzed muropeptides from *N. meningitidis* by reversed-phase high-performance liquid chromatography (HPLC) after peptidoglycan digestion with a muramidase. Indeed, the major peptidoglycan fragments naturally released by Gram-negative bacteria are muropeptides (*14, 15*). This analysis allowed for the separation of muropeptides according to the number of amino acids of the peptidic chain linked to the amino sugars, the degree of polymerization of the peptidic chain, or natural modifications such as O-acetylation or dehydration of the amino sugars (Fig. 2A). Individual muropeptides were collected and tested for their ability to activate the Nod1 pathway. Surprisingly, only two fractions (3 and 17) contained muropeptides able to activate Nod1 (Fig. 2B). Mass spectroscopy analysis revealed that fraction 3 is a muropeptide with a mass/charge ratio *m*/*z* of 893 and the active molecule in fraction 17 is a muropeptide with *m*/*z* of 873 (*16*). The 893 *m*/*z* molecule is consistent with a reduced muropeptide *N*-acetylglucosamine (GlcNAc or "G") β-1,4–linked to *N*-acetylmuramic acid (MurNAc or

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