MOLECULAR AND CELLULAR BIOLOGY, Dec. 1995, p. 6488–6495 Vol. 15, No. 12
0270-7306/95/$04.00+0

DNA Rearrangements in *Euplotes crassus* Coincide with Discrete Periods of DNA Replication during the Polytene Chromosome Stage of Macronuclear Development

JOHN S. FRELS AND CAROLYN L. JAHN*

Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611

Received 8 March 1995/Returned for modification 13 July 1995/Accepted 22 August 1995

Macronuclear development in *Euplotes crassus* begins with polytenization of micronuclear chromosomes and is accompanied by highly precise excision of DNA sequences known as internal eliminated sequences and transposon-like elements (Tecs). Quantitation of radiolabeled-precursor incorporation into DNA indicates that DNA synthesis during formation of polytene chromosomes is not continuous and occurs during two distinct periods. We demonstrate that the timing of Tec excision coincides with these replication periods and that excision can occur during both periods even at a single locus. We also show that Tec and internal eliminated sequence excisions are coincident in the second replication period, thus providing further evidence for similarity in their excision mechanism. Inhibition of DNA synthesis with hydroxyurea diminishes Tec element excision, indicating that replication is an important aspect of the excision process.

Developmentally programmed DNA rearrangements have been identified in a variety of organisms and have been linked to gene regulation and cellular differentiation (4). In most cases, the DNA rearrangements are limited to a particular genetic locus. In contrast, programmed DNA rearrangements in ciliated protozoa occur on a genome-wide scale during the development of a transcriptionally active macronucleus from a transcriptionally silent germ line micronucleus (14, 23, 36). In hypotrichous ciliates like *Euplotes crassus*, the process of macronuclear development is carried to an extreme as specific DNA breakage and rejoining events occur at thousands of sites. The precise nature and magnitude of this genomic reorganization process make *E. crassus* an attractive system for DNA rearrangement studies.

Macronuclear development in *E. crassus* is linked to the sexual phase of the life cycle, when cells of appropriate mating type form conjugating pairs (7). Soon after pairing, haploid micronuclear products of meiosis are exchanged between conjugants and fuse with resident haploid micronuclei to form a new zygotic micronucleus. This nucleus proceeds through two mitotic divisions, resulting in four daughter nuclei. Two of the nuclei will go on to serve as the germ line micronuclei for the daughter cells following the next round of cell division. One of the nuclei will differentiate into the new macronucleus, and one will be degraded. Soon after mitosis, chromosomes within the nucleus destined to differentiate undergo multiple rounds of replication to form polytene chromosomes (reviewed in reference 15). Following polytenization, chromosome bands within the developing macronucleus (also known as the anlagen) become encased in proteinaceous vesicles. During the vesicle stage, the chromosomes are fragmented, telomeres are added to sequences destined to reside in the macronucleus, and the remaining non-macronucleus-destined DNA is degraded. Genomic complexity studies of the related hypotrichs *Stylonychia lemnae* and *Oxytricha nova* have shown that over 90% of the unique DNA sequences and most or all of the repetitive DNA sequences present in the micronucleus are eliminated during macronuclear development (2, 19). In these organisms, the mature macronucleus typically contains over 20,000 different DNA molecules ranging in size from several hundred base pairs up to 20 kbp, each present in approximately 1,000 copies (15).

In *E. crassus*, the best-characterized repetitive sequences are those of the Tec1 and Tec2 transposon-like element families (3, 8–12, 18, 33). Both Tec element families are abundant, together making up 10 to 15% of the micronuclear genome. Tec1 and Tec2 are approximately 5,300 bp in length and similar in overall structure. Tec elements structurally resemble transposons in that they contain long terminal inverted repeats and several conserved open reading frames, including a putative transposase coding region (6, 8), and are flanked by 2-bp (TA) direct repeats that resemble target site duplications. Beginning early in polytenization, Tec elements are precisely excised from the micronuclear DNA to form extrachromosomal circles with the inverted-repeat termini in a head-to-head orientation (9, 18, 33). The termini are joined by a junction sequence in which both direct repeats flank 10 bp of heteroduplex DNA (9, 12). Tec elements frequently interrupt macronucleus-destined sequences; therefore, their precise excision is necessary to generate intact protein-coding regions (3, 10, 18, 33). The identification of conserved open reading frames within both Tec element families led to speculation that Tec-encoded proteins might be involved in the massive excision early in the polytene stage. Transcripts specific for each of the Tec element open reading frames have been detected only in RNA samples from mating cells (11). However, quantitation of the transcripts indicated that they are present at less than 0.001% of the poly(A)$^+$ RNA. While it is possible that the Tec element transcripts support some level of transposition, it seems very unlikely that they are involved in the high level ($>10^5$ copies per cell) of Tec excision. If Tec element-encoded gene products are not responsible for element excision, other DNA-processing mechanisms present in the developing macronucleus must be involved.

* Corresponding author. Mailing address: Department of Cell and Molecular Biology, Northwestern University Medical School, Ward 7-334, 303 East Chicago Ave., Chicago, IL 60611. Phone: (312) 503-2955. Fax: (312) 503-7912. Electronic mail address: jahn@casbah.acns.nwu.edu.

In addition to Tec element interruptions, macronucleus-destined sequences in *E. crassus* are frequently interrupted by short (<500-bp) unique DNA sequences known as internal eliminated sequences (IESs) (3). IESs typically contain short terminal inverted repeats flanked by 5′-TA-3′ direct repeats and are precisely excised late in the polytene stage as circular DNA molecules (3, 31–33). Upon excision, one copy of the direct repeat is retained in the macronucleus-destined sequence and two copies are retained in the excised circle. Like Tec elements, the circles are formed by joining the inverted-repeat termini in a head-to-head orientation. The termini are joined by 10 bases of DNA (6 to 10 bases of which appear to be heteroduplex) that is derived from the flanking micronuclear sequences (16, 31). Thus, the products of IES excision resemble those of Tec excision.

The structural similarities between the circular Tec and IES excision products suggest that the two different types of elements have a common mode of excision. While Tec elements and IESs appear to be excised at different times, both events occur during the polytene stage of macronuclear development. Comparison of data obtained in our laboratory (9) on the timing of Tec element excision relative to the increase in copy number of Tec elements with data obtained by Tausta and Klobutcher (31) on the timing of IES excision relative to changes in the DNA content of the anlagen suggested to us that these excision events may be linked to two consecutive rounds of replication. This was based on the finding that the DNA content of the developing macronucleus increases approximately twofold, from 4 to 8 times the DNA content of the micronucleus, just before the time that Tec element excision is detected, while IES excision occurs about the time that the DNA content increases another twofold, from 8 to 16 times the DNA content of the micronucleus. To gain a better understanding of the relationship between DNA replication and elimination, we have studied the timing of specific rounds of DNA replication during polytene chromosome formation. In this report, we describe the presence of two discrete periods of DNA replication during the polytene chromosome stage of macronuclear development. Elimination of Tecs and IESs occurs during these discrete periods of replication. We show that Tec excision occurs during both DNA replication periods while IES excision occurs only in the second period.

MATERIALS AND METHODS

Cell culture, mating, and DNA preparation. *E. crassus* B6, G12, X1, and X2 were cultured and harvested as previously described (18, 25). Mating was initiated by mixing cultures 3 to 7 days after they had been fed with *Escherichia coli*. Cytological studies were performed on cells isolated throughout the first 24 h postmixing to determine the synchrony of mating, meiosis, and macronuclear development. Cells were fixed in sea salt water by the addition of 2% formaldehyde for 10 min, washed with phosphate-buffered saline, 4′,6-diamidino-2-phenylindole (DAPI) stained, and analyzed by fluorescence microscopy. Total DNA was isolated and purified from starved cells and from mating cells as described previously (9). Briefly, cells were digested with proteinase K and the DNA was purified by phenol and chloroform extractions and ethanol precipitation. RNA was removed by RNase treatment followed by further extraction and precipitation. All samples used in the incorporation studies were examined on agarose gels and shown to be free of RNA prior to scintillation counting. In cases when [^{32}P]TTP was incorporated, the agarose gels were dried and exposed to X-ray film to verify that incorporation was only in the high-molecular-weight DNA expected for labeling of the anlagen.

Cell labeling and drug sensitivity. For radiolabel incorporation studies, mated cells of strains B6 and G12 were labeled with [^{32}P]TTP (600 Ci/mmol) and mated cells of strains X1 and X2 were labeled with [^{3}H]thymidine (60 to 90 Ci/mmol) (both from ICN Biomedical, Irvine, Calif.). Strains X1 and X2 exhibited greater uptake of [^{3}H]thymidine than of [^{32}P]TTP. Mated cells (2 liters, containing approximately 10^7 cells) were concentrated and distributed evenly into small beakers (approximately 10^6 cells in 5 ml). Radiolabel (10 μCi/ml) was added to each beaker sequentially at hourly intervals, after which total DNA from each labeled sample was purified and quantitated. The counts per minute (cpm) of four aliquots of each DNA sample (approximately 10 μg per aliquot) was determined by scintillation counting.

To determine the uptake of [^{3}H]thymidine, triplicate aliquots of cells were labeled for 1 h in an identical manner to that described above. At the end of the labeling, the cells were washed extensively with sea salt water after collection on a 10-μm-pore-size Nitex membrane. The cells were then gently pelleted by centrifugation for 4 min at 100 × *g*, and the pellet was resuspended in 500 μl of 8 M urea. Aliquots (100 μl) were suspended in 3 ml of scintillation fluid (Bio-Safe II; Research Products International Corp., Mt. Prospect, Ill.) for determination of cpm. Identical aliquots of the supernatant from the pelleted cells were counted to ensure that rinsing of the cells had removed the label-containing medium. These counts were <0.1% of the counts in the resuspended cells.

Sensitivity to hydroxyurea was determined by incubating starved cells (5 to 10 cells per microtiter well) with a range of concentrations of hydroxyurea in sea salt water. Each aliquot was examined 24, 48, and 72 h later for increases in the cell population relative to a no-drug control. Hydroxyurea was a potent inhibitor of cell division compared with other drugs tested (aphidicolin and ciclopiroxolamine). At 50 mM hydroxyurea, all of the starting cells were alive and motile after 72 h but little to no increase in the cell number was observed. In contrast, the no-drug controls divided three or four times in the 3-day interval.

Blotting, labeling reactions, hybridizations, and quantitation. Southern blotting was performed as previously described (18). DNA samples were prepared for slot blotting by denaturation in 0.4 N NaOH for 10 min followed by neutralization in 1 M ammonium acetate. Each DNA sample was applied in triplicate onto a Zeta Probe membrane with a Minifold II slot-blotter (Schleicher & Schuell, Keene, N.H.). Prior to hybridization, the DNA was UV cross-linked to the membrane in a Stratalinker 1800 UV cross-linker (Stratagene, La Jolla, Calif.) as recommended by the manufacturer. Plasmid clones that were to be used as hybridization probes to Southern blots or slot blots were radioactively labeled by nick translation and hybridized as described previously (18). Radiolabeled slot blots and dried polyacrylamide gels containing labeled PCR products were exposed on a Fuji imaging plate (type BAS-III), typically for 1 h or less, except in a few instances when slot blots had to be exposed for several hours to produce an image. The images were quantitated on a Fujix BAS 2000 bio-image analyzer (Fuji Film Co., Ltd., Tokyo, Japan). Computer analysis allowed a rectangular area surrounding each band to be quantitated, and the raw radiation dose per given area was expressed as units of photostimulated luminescence.

PCR amplifications. Amplification reactions were performed as previously described (11, 27) with some modifications. All amplification reactions were performed in 50-μl volumes containing 25 pmol of each primer and 1 ng of total *E. crassus* DNA isolated from starved (0 h) cells and from mating cells throughout macronuclear development (referred to as hours postmixing of cells of different mating types). PCR products were labeled during synthesis by the addition of 5 μCi of [^{32}P]dATP (600 Ci/mmol; ICN Biomedical) at the start of the reaction. Other reaction components were 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.02 mM each dGTP, dATP, dTTP, and dCTP. Following the addition of the reaction components described above, all tubes were placed on ice and kept at 4°C during addition of 1 U of *Taq* DNA polymerase, centrifugation, and addition of 50 μl of light mineral oil. Once these steps were completed, the tubes were placed into a programmable thermal cycler (MJ Research) preset at 94°C. Unless otherwise indicated, all PCR amplifications were performed as follows: step 1, 90 s at 94°C; step 2, 60 s at the annealing temperature; step 3, 60 s at 72°C; step 4, 10 s at 94°C; step 5, 10 s at the annealing temperature; step 6, 60 s at 72°C; step 7, cycle back to steps 4 to 6 28 times for a total of 30 cycles; and step 8, decrease to 4°C and stop. The annealing temperature was calculated by subtracting 8°C from the T_m of the primer with the lowest T_m, where the $T_m = 4(GC) + 2(AT)$. Following amplification, reaction products were extracted with chloroform and were loaded on TAE (40 mM Tris acetate, 1 mM EDTA)–6% polyacrylamide gels. The gels were electrophoresed for 3 h at 35 mA and 50 W with TAE as the running buffer. They were then fixed and dried by standard procedures (28).

The quantitative nature of the PCR experiment (see Fig. 5) was determined as follows. First, PCR was performed with total DNA isolated from starved *E. crassus* cells in combination with primers OP157 and OP158. Aliquots were removed from the reaction at the end of cycles 25 through 30, and products were resolved as described above and quantitated by phosphoimaging. The results indicated that by 30 cycles, the reactions were still within the exponential amplification phase (data not shown). Therefore, it appeared that under the conditions described above, 30 cycles of PCR was sufficient to obtain quantitative results. Second, PCR was performed with a series of twofold dilutions of an EC2 plasmid clone in combination with primers OP157 and OP158. Twofold increases in the amount of starting template were detected as twofold increases in the amount of the specific PCR product (data not shown). This relationship held up until starting template concentrations exceeded 1,000 copies per reaction. Since the target sequence in *E. crassus* is unlikely to exceed 1,000 copies per reaction with 1 ng of total DNA during amplification in the polytene stage, the results described in Fig. 5 should accurately reflect the copy number of the locus in vivo.

RESULTS

Synchrony of pairing and macronuclear development in two different combinations of *E. crassus* strains. Our ability to detect S phases by labeled nucleotide incorporation was dependent on the synchrony of the cells as they proceeded through macronuclear development. Two separate combinations of *E. crassus* strains were used during these studies, strains B6 and G12 and strains X1 and X2. Strains B6 and G12 are exconjugant lines derived from a pooled mating as described previously (9), and they proceed through macronuclear development with the same timing and degree of synchrony as the previously described strains 5 and 25 and strains 8 and 24 (9). During the course of these studies, cultures of B6 and G12 began to exhibit signs of senescence and eventually failed to synchronously mate and proceed through macronuclear development. As a result, additional strains were derived from exconjugants. Strains X1 and X2 exhibited the highest level of mating synchrony of all the newly derived strains and were therefore used in all subsequent studies. Comparisons of developmental timing between strains B6 and G12 and strains X1 and X2 yielded surprising results. The timing of pair formation differs greatly between the two different combinations of mating cells (Fig. 1A). Within 2 h of mixing of strains X1 and X2, more than 50% of the cells form pairs, and by 4 h, nearly 100% of the cells are paired. Strains B6 and G12 require 4 to 5 h to achieve 50% pairing, and they reach a maximum pairing of 70% by 7 h. The percentage of mated cells containing a visually detectable developing macronucleus (referred to as the anlagen) was determined for each combination of strains (Fig. 1B). In strains X1 and X2, approximately 30% of the mated cells had visually detectable anlagen by 15 h postmixing and nearly 100% of the mated cells contained anlagen by 18 h postmixing. In contrast, in strains B6 and G12, approximately 90% of the mated cells contained anlagen by 23 h postmixing. Thus, both pairing and anlage appearance occur 3 to 4 h sooner in strains X1 and X2 than in strains B6 and G12. The developmental timing of pairing and anlage formation for either combination of strains has not changed over the lifetime of these strains (2 years for B6 and G12 and 1.5 years for X1 and X2, to date). However, the percentage of cells exhibiting pairing and anlage formation declined with the age of the B6 and G12 strains.

The timing of Tec element excision in strains X1 and X2 was determined by hybridization of a [^{32}P]dCTP-labeled plasmid Tec clone to Southern-blotted total DNA isolated from cells at various times postmixing. Tec excision is detected by the presence of the extrachromosomal circular forms of the element (9). In strains X1 and X2, extrachromosomal forms are usually detectable by 22 h postmixing (Fig. 1C). In strains B6 and G12, Tec excision is detected between 26 and 28 h postmixing as previously described (11). The difference in timing of Tec excision between strains corresponds well to the timing differences in pairing and anlage formation described above. These differences in developmental timing indicate that mating reactivity is a major determinant of the timing of subsequent developmental events. Since the mating reaction typically takes 3 h, S phases shorter than this would most probably not be detected as distinct peaks of incorporation.

Identification of discrete periods of DNA replication. To identify periods of DNA replication during polytene chromosome formation, cells undergoing macronuclear development were labeled with either [^{32}P]TTP (for strains B6 and G12) or [^{3}H]thymidine (for strains X1 and X2). Cells were labeled for 1-h periods during the initial 25 to 30 h of anlage development corresponding to the polytene stage. Following each labeling period, cells were harvested, total DNA was isolated, and the

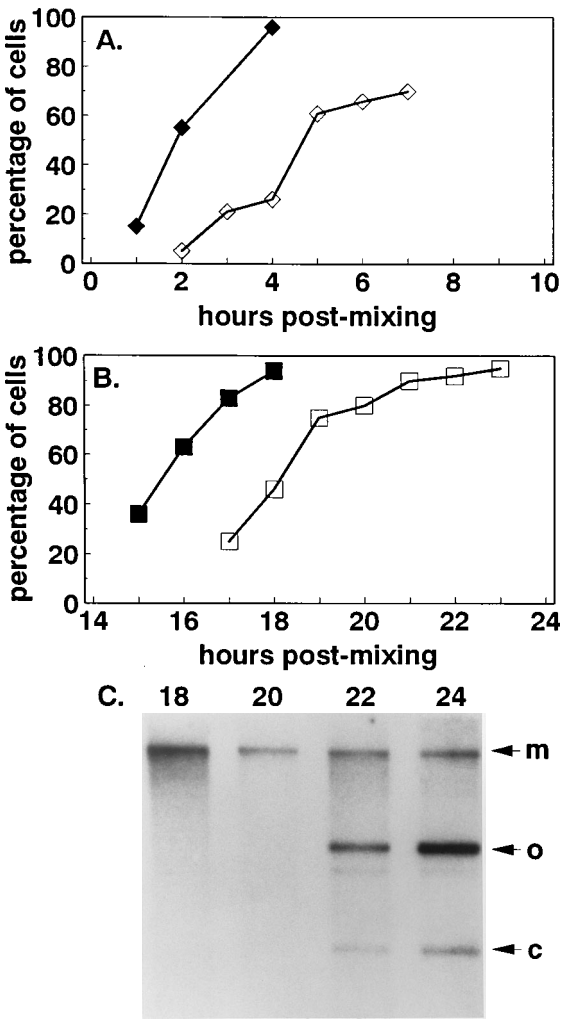

FIG. 1. Comparison of synchrony of pairing and macronuclear development between strains B6 and G12 and strains X1 and X2. After mixing of both combinations of strains, aliquots were removed hourly from the cultures of mating cells and processed for microscopy. (A) Percentage of cells that formed pairs in both strain combinations by 7 h postmixing. Solid diamonds, strains X1 and X2; open diamonds, strains B6 and G12. (B) Percentage of mated cells that had visible anlagen in both strain combinations from 15 to 23 h postmixing. Solid squares, strains X1 and X2; open squares, strains B6 and G12. (C) Detection of Tec excision in strains X1 and X2 by Southern blotting and hybridization. Tec excision is detected by 22 h postmixing by the appearance of the extrachromosomal supercoiled (c) and open circular (o) forms of the element. The chromosomal main band is also indicated (m).

cpm per nanogram was calculated. Strains B6 and G12 exhibited at least two discrete periods of incorporation in the 20- to 45-h time interval (Fig. 2A). One period of incorporation peaks at 24 to 26 h postmixing. Incorporation declines until after 36 h, when it increases again. The data shown in Fig. 2A are derived from an experiment with one set of cells mated at various intervals within a 12-h period. In multiple repetitions of this experiment, cells were mated and labeled at 1-h intervals for 10-h periods that overlapped the 25-h experiment whose results are shown in Fig. 2A. Although there were quantitative differences (up to twofold) in the amount of label incorporated in different experiments, the qualitative results of increases and decreases in specific activity were reproducible within each experiment. For instance, two repetitions were carried out for the periods from 20 to 30 h and from 25 to 35

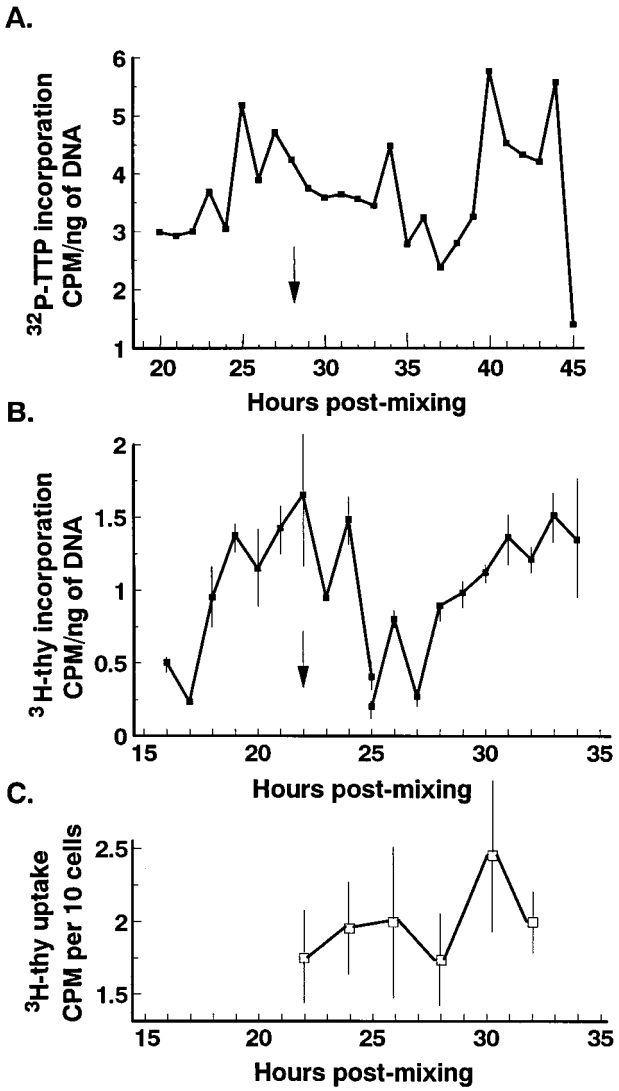

FIG. 2. Discrete periods of DNA replication during the polytene stage. Mating cells were labeled sequentially at hourly intervals from 16 to 36 h postmixing, after which total DNA was isolated from each of the samples. Each point represents the average of four replicate cpm determinations of equal amounts of a single labeled total DNA sample (error bars show the standard deviation of the replicate cpm determinations). The values are expressed as the cpm per nanogram of total DNA for each labeling period. (A and B) Discrete periods of DNA replication were detected in strains B6 and G12 by [^{32}P]TTP incorporation (A) and strains X1 and X2 by [^{3}H]thymidine incorporation (B). Vertical arrows in panels A and B indicate the earliest time at which extrachromosomal forms of Tec elements are detected. (C) Uptake of [^{3}H]thymidine as determined for mated X1 and X2 cells. The labeling was identical to that in panel B. Uptake was measured by determining the cpm remaining in the cells after extensive washing (see Materials and Methods). The cpm was determined on multiple aliquots with approximately 10^5 cells per aliquot. Error bars represent the standard deviation of three independent labelings of equivalent numbers of cells (see Materials and Methods).

h with the B6 and G12 strains. These experiments showed a decrease in the specific activity (cpm per nanogram) starting at 28 or 30 h. Similar repetitions for the later period showed a increase in incorporation after 40 h.

Strains X1 and X2 also exhibited discrete peaks of label incorporation (Fig. 2B). A broad period of incorporation between 17 and 24 h coincides with Tec excision at 22 h postmixing in these strains (Fig. 1C). A second period of label incorporation begins at 28 h and continues until at least 35 h. The broad nature of this peak also suggests that more than one round of replication is occurring. This second peak of incorporation resembles the broad period of incorporation from 38 to 45 h in the mated B6 and G12 strains. As observed with the B6 and G12, multiple repetitions of the labeling showed quantitative differences in incorporation between experiments, but a decrease in incorporation was always observed immediately after the time that extrachromosomal Tec elements were detected. Thus, both combinations of strains showed a similar pattern of two discrete periods of DNA replication during the polytene chromosome phase of macronuclear development.

To determine whether differences in incorporation could be explained by differences in the uptake of thymidine in cells at different stages of development, we quantitated the uptake of [^{3}H]thymidine in parallel with incorporation into DNA for strains X1 and X2 (see Materials and Methods) (Fig. 2C). This demonstrated that uptake of thymidine does not change during the period that incorporation into DNA decreases. On a per cell basis, the amount of [^{3}H]thymidine taken up by the cells is approximately 20-fold higher than the amount incorporated into DNA. Thus, we believe that differences in incorporation are due to differences in DNA synthesis.

Inhibition of Tec excision with hydroxyurea. The above results indicate that Tec excision is associated with a discrete period of DNA replication; therefore, we wanted to determine whether DNA replication is required for Tec element excision. To test this, cells of strains X1 and X2 undergoing macronuclear development were incubated with or without the DNA replication inhibitor hydroxyurea and tested for differences in Tec element copy number, overall [^{3}H]thymidine incorporation, and the level of extrachromosomal circle produced. Changes in Tec element copy number were monitored by quantitating the Tec-to-actin-gene ratio by slot blot hybridization. Because of the high copy number of the actin gene in the macronucleus, the amount of actin gene hybridization in total DNA remains relatively constant throughout development (data not shown). Therefore, quantitating the level of actin gene hybridization provides a good internal standard to control for sample loading.

Mating cells were incubated with or without 50 mM hydroxyurea from 18 to 23 h postmixing. Total DNA from the plus-drug and no-drug samples was purified and analyzed. Incubation with hydroxyurea from 18 to 23 h decreased the amount of [^{3}H]thymidine incorporation by approximately 45% (Fig. 3A). The replication of Tec element DNA sequences (as measured by changes in the Tec to actin ratio) was inhibited by about 35% (Fig. 3B). The amount of excised extrachromosomal Tec elements was markedly decreased in samples treated with hydroxyurea, as shown in the 23-h sample (Fig. 3C). The addition of hydroxyurea after 18 h resulted in little to no detectable changes in Tec replication or circle formation (data not shown). We believe that this is due to the mechanism of action of hydroxyurea. Hydroxyurea is a potent inhibitor of ribonucleotide reductase and inhibits DNA synthesis by blocking the production of deoxyribonucleotides (17, 20). Therefore, the presence of large intracellular pools of deoxynucleoside triphosphates could mask the effects of hydroxyurea until their depletion. Our results suggest that depletion of the pools may require 4 to 5 h in *E. crassus* cells undergoing macronuclear development. We have attempted these experiments with other DNA replication inhibitors that should be more rapid in their action (aphidicolin and ciclopiroxolamine) but were unable to achieve greater than 30% inhibition of DNA replication on the basis of assays of radiolabelled-nucleotide incorporation. This level of inhibition made the detection of

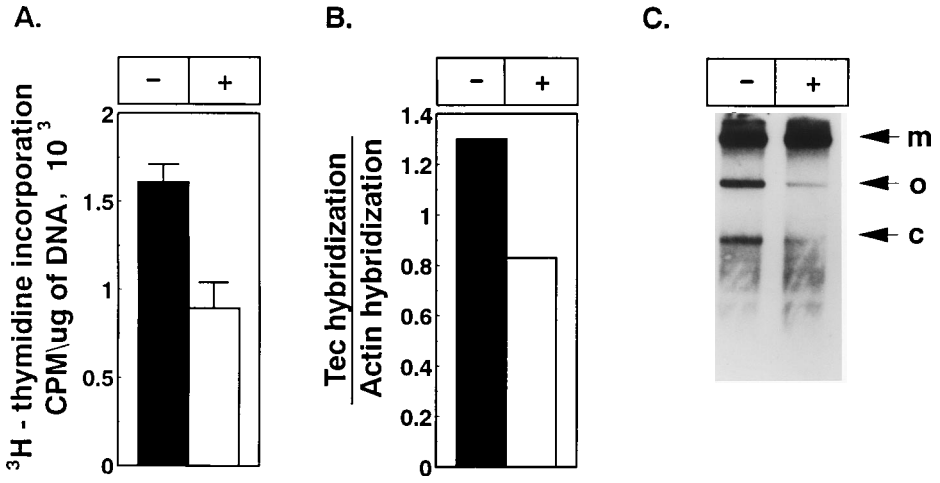

FIG. 3. Inhibition of DNA replication and Tec excision in strains X1 and X2 by hydroxyurea. (A) Mating cells were incubated with [^{3}H]thymidine (10 μCi/ml), with or without 50 mM hydroxyurea, from 18 to 23 h postmixing. Cells were harvested at 23 h, total DNA was purified, and the cpm per microgram of total DNA for each sample was determined. (B) The 23-h with-drug and no-drug samples were slot blotted (5 μg per slot), and the Tec-to-actin-gene DNA sequence ratio was determined by hybridization and quantitation. The average of Tec-to-actin-gene ratio values for triplicate slots of each sample was determined. (C) The 23-h with-drug and no-drug samples were electrophoresed, Southern blotted, and hybridized with a labeled Tec-specific clone. Hybridization to extrachromosomal Tec is designated as supercoiled (c) or open circular (o). m, chromosomal main band.

effects on Tec element copy number and circle formation ambiguous.

Identification of a second round of Tec excision. To further confirm that Tec elements in strains X1 and X2 are undergoing replication during the interval of their excision, we monitored changes in the Tec-to-actin-gene ratio 18 to 36 h postmixing, by slot blot hybridization with total DNA isolated from mating cells (Fig. 4). Identical experiments performed with strains B6 and G12 yielded results similar to those previously reported for strains 5 and 25 and strains 8 and 24 (9). In mated X1 and X2 cells, the increase in Tec element copy number at 20 h follows the timing of the incorporation peak shown in Fig. 2B. Tec excision at 22 h results in a loss of Tec sequences, presumably because of degradation of the extrachromosomal forms (9). A second increase in Tec element copy number beginning at 30 h coincides with the second broad period of label incorporation, also shown in Fig. 2B. By 36 h, the Tec element copy number begins to decrease as it did following excision at 22 h. This suggests that there is a second round of Tec excision during the second period of DNA replication in the anlagen.

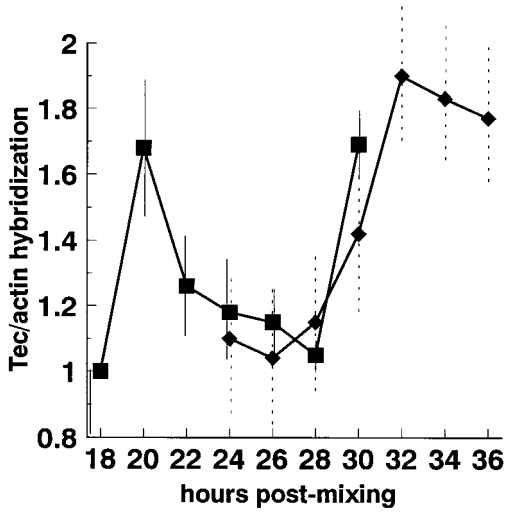

FIG. 4. Identification of a second round of Tec element amplification and excision. Total DNA was isolated from two separate batches of mating cells, 18 to 36 h postmixing. The samples were slot blotted (5 μg per slot), and the Tec-to-actin-gene DNA sequence ratio was determined by hybridization and quantitation. The average of Tec-to-actin-gene ratio values for triplicate slots of each sample was determined. The resulting values were plotted relative to the 18-h Tec-to-actin-gene-ratio value.

PCR analysis of amplification and excision patterns during the polytene stage. The above results showing two rounds of increases and decreases in Tec copy number could reflect amplification events at two different sets of Tec integration sites and/or the behavior of Tecs within the multiple polytene strands of each integration site. To investigate this further, we monitored Tec amplification and excision at a single locus by PCR. As described in Materials and Methods, we have developed a PCR assay that allows us to visualize copy number differences in sequences in time course DNA samples assayed in parallel. We studied a Tec integrated at a locus isolated in the previously described EC2 micronuclear clone (10, 18). This clone contains homology to several macronuclear molecules. Two of these macronucleus-destined sequences (sequences containing homology to 2.7- and 0.85-kb macronuclear molecules) are arranged immediately adjacent to each other. During macronuclear development, the chromosome is precisely fragmented between these two sequences. Therefore, the sequence organization at the junction of the 2.7- and 0.85-kb macronucleus-destined sequences is not present in the mature macronucleus. Primers specific for each side of this fragmentation site (OP157 and OP158 [Fig. 5A]) allow the generation of a 240-bp PCR product specific for the EC2 junction sequence within the micronucleus and the anlagen. Previous studies have shown that the DNA content of the micronuclei in mating cells does not change during this time interval (32). Therefore, any increases in the amount of PCR product can be attributed solely to increases in the DNA content of the anlagen. The 0.85-kb macronucleus-destined sequence contained within EC2 is interrupted by two Tec2 elements, one inserted inside the other. Previous experiments have shown that these Tec elements are precisely excised early in the polytene stage (18). A primer specific for the head of the Tec2 inverted repeat (OP163; Fig. 5A) was designed so that it would prime DNA synthesis out of the element and into the adjacent macronu-

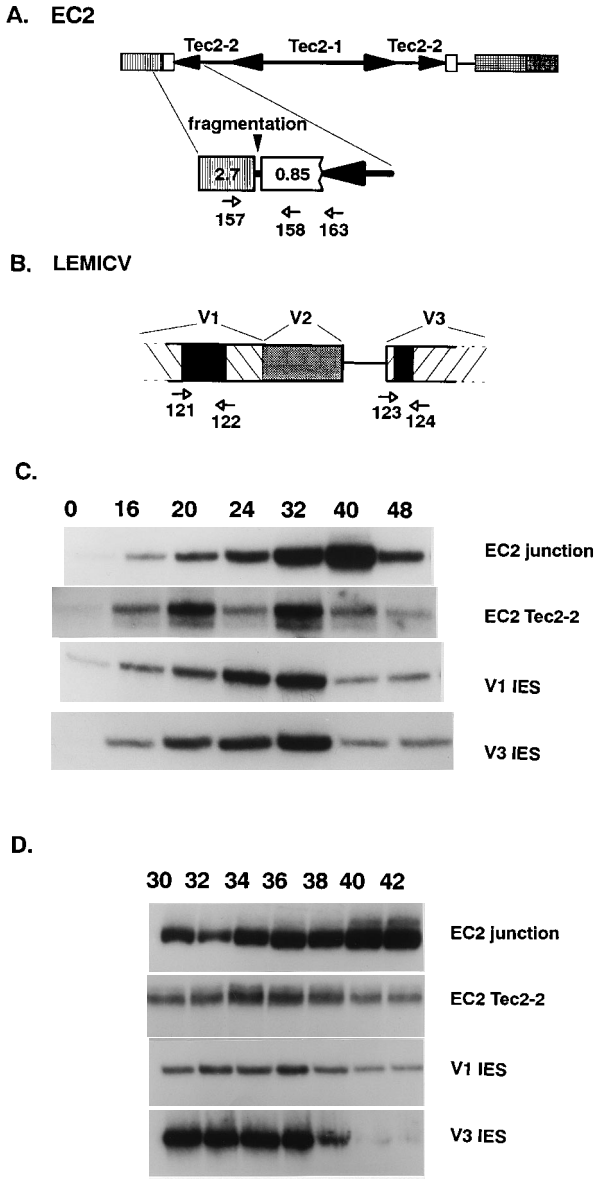

FIG. 5. PCR analysis of specific DNA sequence amplification patterns during the polytene stage. (A) A region of the previously characterized EC2 micronuclear phage clone (18). EC2 contains homology to several macronucleus-destined sequences, indicated as hatched and open boxes. A 0.85-kb macronucleus-destined sequence (open box) is interrupted by two Tec2 elements, one inserted inside the other. A 2.7-kb macronucleus-destined sequence (vertically hatched box) is adjacent to the 0.85-kb sequence. Following amplification during the polytene stage, the chromosome is fragmented precisely between these two sequences. The oligonucleotide primers OP157 and OP158 anneal within the 2.7- and 0.85-kb macronucleus-destined sequences, respectively. PCR amplifications containing OP157, OP158, and total DNA give rise to a 240-bp product that is specific for the developing macronucleus and the micronucleus. The oligonucleotide primers OP157 and OP163 anneal within the 2.7-kb macronucleus-destined sequence and the head of the Tec2-2 inverted repeat, respectively. PCR amplifications containing total DNA, OP157, and OP163 generate a 400-bp product that is specific to the developing macronucleus and the micronucleus. (B) A region of the previously characterized micronuclear clone LEMICV (3). LEMICV contains several macronucleus-destined sequences, V1, V2, and V3. The V1 and V3 macronucleus-destined sequences are interrupted by 371- and 144-bp IESs, respectively. The positions of the V1-specific oligonucleotide primers OP121 and OP122 and the V3-specific oligonucleotide primers OP123 and OP124 are shown. PCR amplifications with total DNA and either OP121/OP122 or OP123/OP124 detect only the integrated (unexcised) V1 and V3 IESs, respectively. (C) Amplification and excision patterns of Tecs and IESs. Total DNA samples isolated at various intervals from mating cells (strains X1 and X2) 0 to 48 h postmixing were used in PCR amplifications with OP157 and OP158 (EC2 junction), OP157 and OP163 (EC2 Tec2-2 element), OP121 and OP122 (V1 IES), and OP123 and OP124 (V3 IES). (D) Coincidence of Tec and IES excision. PCR amplifications identical to those in panel C were carried out on total DNA samples isolated at 2-h intervals between 30 and 42 h after mixing of X1 and X2 cells.

cleus-destined sequence. The primer combination of OP163 and OP157 (Fig. 5A) could then be used to generate a 400-bp PCR product specific for the integrated (unexcised) Tec2 locus.

PCR was performed with each primer combination by using 1 ng of total DNA isolated from starved cells (0 h) and from mating cells throughout the polytene stage. The products were labeled with [^{32}P]dATP during synthesis, electrophoresed on 6% polyacrylamide gels, and visualized by autoradiography as shown in Fig. 5C and D. The EC2 junction exhibited amplification, as indicated by the steady pattern of increase in the amount of PCR product, until 48 h postmixing (Fig. 5C). By 48 h, chromosome fragmentation at this locus results in a sharp decrease in PCR product formation. The amplification and excision pattern of the EC2 Tec2-2 element is shown in Fig. 5C. The first round of Tec excision occurs between 20 and 24 h and is detected by a decrease in the amount of PCR product. An additional round of amplification is evident by 32 h and is followed by excision detected as a decrease in the amount of the PCR product by 40 h. The amount of product decreases even further by 48 h, presumably as a result of fragmentation at the locus.

PCR analysis of IES excision. The identification of a specific Tec element that excises during at least two different rounds of replication prompted us to determine the timing of IES excision in strains X1 and X2. Two specific IESs, V1 and V3, have previously been identified and characterized in great detail (3, 33). The V1 IES (371 bp) and the V3 IES (144 bp) interrupt the V1 and V3 macronucleus-destined sequences represented in the micronuclear phage clone LEMICV as shown in Fig. 5B. Previous studies have shown that both V1 and V3 are precisely excised between 45 and 50 h postmixing (32). Since strains X1 and X2 are accelerated in their developmental timing, it seemed possible that the timing of IES excision would be quite different in these strains. The timing of excision of both IESs was determined by PCR with oligonucleotide primers that span the boundaries of the IESs (V1: OP121, OP122; and V3: OP123, OP124 [Fig. 5B]) such that only the DNA strands that contain an integrated IES can serve as a template during PCR. PCR was performed with each primer combination, using 1 ng of each total DNA sample as described above. As shown in Fig. 5C, both the V1 and V3 IESs appear to excise between 32 and 40 h postmixing, as indicated by the sudden decrease in PCR product formation after 32 h. Therefore, the timing of IES excision in strains X1 and X2 is much earlier than the timing described for previously used strains and appears to coincide with the second round of Tec excision described above. The coincidence of these excision events is further demonstrated by PCR carried out on total DNA samples isolated every 2 h between 30 and 42 h after mixing of strains X1 and X2 (Fig. 5D). The EC2 Tec2-2 and V1 and V3 IESs show identical patterns of decreased products at 36 to 40 h. In this set of DNA samples, the copy number of the EC2 junction continues to increase after 40 h and fragmentation is not apparent until after 46 h (data not shown). Thus, the decreased amounts of products for the EC2 Tec2-2 and IESs cannot be due to DNA degradation at the time of fragmentation and most probably are due to excision events.

DISCUSSION

Discrete periods of DNA replication occur during polytene chromosome formation and Tec element excision. Radiolabel incorporation assays have allowed the identification of two

discrete periods of DNA replication during the formation of polytene chromosomes in *E. crassus*. We have not identified single S phases corresponding to one replication of the entire genome. However, we see a distinct pause dividing two major periods of replication. The observance of discrete periods of DNA replication during polytene chromosome formation is similar to what is observed in *Drosophila melanogaster*. Polytene cells from *D. melanogaster* incorporate [^{3}H]thymidine during discrete intervals (26, 29). These intervals indicated the separation of discrete S phases in which part or all of the genome is replicated. The timing of discrete periods of DNA replication in *E. crassus* coincides with the detection of Tec element excision in mating cells from different combinations of strains which differ in their timing of macronuclear development. This suggests that the synthesis or activation of the enzymatic machinery responsible for Tec excision may be linked to the polytene-stage S phases within developing macronuclei. The inhibition of Tec excision by the DNA replication inhibitor hydroxyurea further indicates that DNA replication is somehow required for Tec element excision.

Tec excision occurs during more than one round of DNA replication. Previous work with strains similar in developmental timing to B6 and G12 led to the conclusion that Tec elements were completely excised during a 2- to 4-h period (9, 18), because the amount of circular products remained constant or declined after 30 h. However, these studies did not monitor increases or decreases in Tec element copy number throughout the entire polytene stage, as shown in Fig. 4 for the X1 and X2 strains. The increase and decrease in Tec element copy number following the excision detected at 22 h (Fig. 4) indicates that not all Tec elements are excised at once and that a substantial population of Tec elements are replicated in the second replication period.

The PCR experiment described in Fig. 5C demonstrates that multiple rounds of Tec excision occur at a single locus. Copies of the Tec element at the EC2 locus are still present and have been replicated following the excision at 22 h. By 40 h, these Tec elements have gone through another round of excision. The association of Tec excision with more than one round of DNA replication indicates that Tec excision is not necessarily confined to a 2- to 4-h developmental window. Instead, these data suggest that Tec elements can continue to be excised during subsequent rounds of replication.

Tec excision and IES excision occur at the same time. The previously characterized V1 and V3 IESs were shown to be excised between 40 and 50 h postmixing in cell lines that proceeded through macronuclear development with timing similar to that of B6 and G12 (32). The results of the PCR experiment illustrated in Fig. 5 demonstrate that the V1 and V3 IESs are excised between 36 and 40 h postmixing in strains X1 and X2. The timing of this excision coincides with several other events described above: a broad peak of [^{3}H]thymidine incorporation (Fig. 2B), a twofold increase in the copy number of the EC2 macronuclear junction (Fig. 5), and the second detectable round of Tec excision (Fig. 4 and 5). Thus, it appears that Tec and IES excision can occur simultaneously during a period of DNA replication. Taken together with the structural similarities of their excision products, these results further indicate that the excision of both types of elements may be mediated by the same enzymatic machinery.

While Tecs and IESs may share the same basic excision mechanism, the different overall timing of Tec and IES excision suggests that each type of element is targeted differently for excision. Tec excision is initially detected at approximately 22 h postmixing, at least 10 h earlier than the detection of V1 and V3 IES excision. Since only the V1 and V3 IESs were tested in this study, it is not clear whether their timing of excision is representative of all IESs. In light of the Tec element excision findings, it seems possible that IESs are also excised during multiple periods of DNA replication. Likewise, some Tec elements may be excised only in the second period of replication, as seen for the two IESs analyzed. Further studies of individual Tec insertion sites and other IESs by the PCR strategies described here may clarify what timing differences exist.

Possible roles of DNA replication in DNA elimination. The developmentally regulated, precise DNA elimination events in *E. crassus* could be linked to DNA replication in several different ways. A direct relationship between these two processes is possible if specific sequences are excised during or after passage of the DNA replication fork. For example, one of the first proposed models of IES excision involved a "slipped-mispairing" mechanism in which DNA synthesis across one of the direct repeats could allow slippage of the nascent strand and pairing with the direct repeat on the other side of the IES (24). The newly replicated DNA strands would contain heteroduplex DNA consisting of a looped-out IES that could then be excised by DNA repair enzymes. For the Tec elements, strand separation during replication would allow intrastrand pairing of the large inverted repeats, which would greatly facilitate deletion. Similar models have been proposed to explain the transposase-independent precise excision of Tn*10* (13, 21). However, this model does not readily explain the existence of the double-stranded circular products of Tec and IES excision and their junction structure. Thus, a direct breakage-and-rejoining mechanism has been favored (12, 16). Nevertheless, large-inverted-repeat interaction during DNA replication may play a role in Tec excision that leads to the early excision of Tecs relative to IESs, which have little, if any, inverted repeat homology at their ends.

In addition to the above arguments concerning the circular products, the timing of Tec and IES excision relative to periods of DNA replication suggests that excision is not an immediate result of DNA replication. Excision occurs during the second half of the replication periods we detect. Likewise, the rise and fall in Tec copy number indicates that replication results in at least a twofold increase in copy number before excision occurs. Thus, it appears that most of the replication is complete before excision. Although this could be a function of the timing of a postreplication repair process, other indirect links between replication and excision come to mind. DNA elimination could be indirectly related to replication if the deposition of developmental stage-specific chromosomal proteins during DNA synthesis facilitates the excision of specific DNA sequences. Studies of *Tetrahymena* chromosomal proteins indicate that macronucleus-specific histones and nonhistone proteins such as high-mobility-group proteins are deposited into premeiotic micronuclei and developing macronuclei (1, 5, 30, 34). Changes in such chromosomal proteins could induce or stabilize unusual DNA structures and facilitate DNA breakage and rejoining events. Furthermore, chromatin remodeling to generate a transcriptionally active gene can require binding of proteins during DNA replication while histones are released from the DNA (22, 35). The assembly of transcriptionally active chromatin in the developing macronucleus may require DNA replication and may play a role in targeting specific sequences for elimination versus retention. If the buildup of transcriptionally competent structures is required for the excision of Tecs and IESs, we might expect variability between loci depending on how rapidly transcriptional activation complexes assemble at different sites. Finally, another indirect relationship with replication could be a consequence of S-phase-specific regulation of gene

expression. Thus, expression of transcripts and appearance of proteins making up the Tec and IES excision machinery could be linked to replication periods.

ACKNOWLEDGMENTS

This work was supported by NSF grant MCB-9319009 to C.L.J. and by the Lucille P. Markey Charitable Trust.

REFERENCES

1. **Allis, C. D., and J. C. Wiggins.** 1984. Histone rearrangements accompany differentiation and dedifferentiation in *Tetrahymena*. Dev. Biol. **101:**282–294.
2. **Ammermann, D., G. Steinbrück, L. von Berger, and W. Hennig.** 1974. The development of the macronucleus in the ciliated protozoan *Stylonychia mytilus*. Chromosoma **45:**401–429.
3. **Baird, S. E., G. M. Fino, S. L. Tausta, and L. A. Klobutcher.** 1989. Micronuclear genome organization in *Euplotes crassus*: a transposon-like element is removed during macronuclear development. Mol. Cell. Biol. **9:**3793–3807.
4. **Berg, D. E., and M. M. Howe (ed.).** 1989. Mobile DNA. American Society for Microbiology, Washington, D.C.
5. **Chicoine, L. G., D. W. Wenkert, R. Richman, J. C. Wiggins, and C. D. Allis.** 1984. Modulation of H1-like histones during macronuclear development in *Tetrahymena*: selective elimination of linker histones during the differentiation of new macronuclei. Dev. Biol. **109:**1–8.
6. **Doak, T. G., F. P. Doerder, C. L. Jahn, and G. Herrick.** 1994. A proposed superfamily of transposase genes: transposon-like elements in ciliated protozoa and a common "D35E" motif. Proc. Natl. Acad. Sci. USA **91:**942–946.
7. **Heckmann, K.** 1964. Experimentalle untersuchungen an *Euplotes crassus*. I. Paarungssystem, Konjugation, und Determination der Paarungstypen. Z. Vererbungsl. **95:**114–124.
8. **Jahn, C. L., S. D. Doktor, J. S. Frels, J. W. Jaraczewski, and M. F. Krikau.** 1993. Structures of the *Euplotes crassus* Tec*1* and Tec*2* elements: identification of putative transposase coding regions. Gene **133:**71–78.
9. **Jahn, C. L., M. F. Krikau, and S. Shyman.** 1989. Developmentally coordinated en masse excision of a highly repetitive element in *E. crassus*. Cell **59:**1009–1018.
10. **Jahn, C. L., L. A. Nilles, and M. F. Krikau.** 1988. Organization of the *Euplotes crassus* micronuclear genome. J. Protozool. **35:**590–601.
11. **Jaraczewski, J. W., J. S. Frels, and C. L. Jahn.** 1994. Developmentally regulated, low abundance Tec element transcripts in *Euplotes crassus*-implications for DNA elimination and transposition. Nucleic Acids Res. **22:**4535–4542.
12. **Jaraczewski, J. W., and C. L. Jahn.** 1993. Elimination of Tec elements involves a novel excision process. Genes Dev. **7:**95–105.
13. **Kleckner, N.** 1989. Transposon Tn*10*, p. 227–268. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
14. **Klobutcher, L. A., and C. L. Jahn.** 1991. Developmentally controlled genomic rearrangements in ciliated protozoa. Curr. Opin. Genet. Dev. **1:**397–403.
15. **Klobutcher, L. A., and D. M. Prescott.** 1986. The special case of the hypotrichs, p. 111–154. *In* J. Gall (ed.), The molecular biology of ciliated protozoa. Academic Press, Inc., New York.
16. **Klobutcher, L. A., L. R. Turner, and J. LaPlante.** 1993. Circular forms of developmentally excised DNA in *Euplotes crassus* have a heteroduplex junction. Genes Dev. **7:**84–94.
17. **Kornberg, A.** 1980. DNA replication. W. H. Freeman & Co., San Francisco.
18. **Krikau, M. F., and C. L. Jahn.** 1991. Tec2: a second transposon-like element demonstrating developmentally coordinated excision in *Euplotes crassus*. Mol. Cell. Biol. **11:**4751–4759.
19. **Lauth, M. R., B. B. Spear, J. Heumann, and D. M. Prescott.** 1976. DNA of ciliated protozoa: DNA sequence diminution during macronuclear development of Oxytricha. Cell **7:**67–74.
20. **Levenson, V., and J. L. Hamlin.** 1993. A general protocol for evaluating the specific effects of DNA replication inhibitors. Nucleic Acids Res. **21:**3997–4004.
21. **Lundblad, V., A. Taylor, G. Smith, and N. Kleckner.** 1984. Unusual alleles of RecB and RecC stimulate excision of the inverted repeat transposons Tn*10* and Tn*5*. Proc. Natl. Acad. Sci. USA **81:**824–828.
22. **Paranjape, S. M., R. T. Kamakaka, and J. T. Kadonaga.** 1994. Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. **63:**265–297.
23. **Prescott, D. M.** 1992. The unusual organization and processing of genomic DNA in hypotrichous ciliates. Trends Genet. **8:**439–445.
24. **Ribas-Aparicio, R. M., J. J. Sparkowski, A. E. Proulx, J. D. Mitchell, and L. A. Klobutcher.** 1987. Nucleic acid splicing events occur frequently during macronuclear development in the protozoan *Oxytricha nova* and involve the elimination of unique DNA. Genes Dev. **1:**323–336.
25. **Roth, M. R., M.-Y. Lin, and D. M. Prescott.** 1985. Large scale synchronous mating and the study of macronuclear development in *Euplotes crassus*. J. Cell Biol. **101:**79–84.
26. **Rudkin, G. T.** 1972. Replication in polytene chromosomes, p. 50–85. *In* W. Beerman, H. Ursprung, and J. Reinert (ed.), Results and problems in cell differentiation: developmental studies with giant chromosomes, vol. 4. Springer-Verlag, New York.
27. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Enzymatic amplification of beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Science **239:**487–491.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Spradling, A., and T. Orr-Weaver.** 1987. Regulation of DNA replication during *Drosophila* development. Annu. Rev. Genet. **21:**373–403.
30. **Stargell, L. A., J. Bowen, C. A. Dadd, P. C. Dedon, M. Davis, R. G. Cook, C. D. Allis, and M. A. Gorovsky.** 1993. Temporal and spatial association of histone H2A variant hv1 with transcriptionally competent chromatin during nuclear development in *Tetrahymena thermophila*. Genes Dev. **7:**2641–2651.
31. **Tausta, S. L., and L. A. Klobutcher.** 1989. Detection of circular forms of eliminated DNA during macronuclear development in *Euplotes crassus*. Cell **59:**1019–1026.
32. **Tausta, S. L., and L. A. Klobutcher.** 1990. Internal eliminated sequences are removed prior to chromosome fragmentation during development in *Euplotes crassus*. Nucleic Acids Res. **18:**845–853.
33. **Tausta, S. L., L. R. Turner, L. K. Buckley, and L. A. Klobutcher.** 1991. High fidelity developmental excision of Tec*1* transposons and internal eliminated sequences in *Euplotes crassus*. Nucleic Acids Res. **19:**3229–3236.
34. **Wang, T., and C. D. Allis.** 1993. An abundant high-mobility-group-like protein is targeted to micronuclei in a cell-cycle-dependent and developmentally regulated fashion in *Tetrahymena thermophila*. Mol. Cell. Biol. **13:**163–173.
35. **Wolffe, A. P.** 1991. Implications of DNA replication for eukaryotic gene expression. J. Cell Sci. **9:**201–206.
36. **Yao, M.-C.** 1989. Site-specific chromosome breakage and DNA deletion in ciliates, p. 715–734. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.