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Internal eliminated sequences are removed prior to chromosome fragmentation during development in *Euplotes crassus*

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ABSTRACT

The hypotrichous ciliated protozoa undergo a massive genome rearrangement process after their sexual cycle. One frequent type of rearrangement is the removal of DNA sequences (internal eliminated sequences; IESs) from internal regions of DNA molecules. In this study, we characterized the removal of IESs in *Euplotes crassus*. Southern hybridization analyses combined with cytological observations indicated that IES removal is an early event in macronuclear development, occurring during the polytene chromosome stage and prior to the chromosome fragmentation process. The results are consistent with IES removal occurring via an intramolecular DNA breakage and rejoining process.

INTRODUCTION

Genomic reorganization occurs as a normal, developmentally regulated process in a variety of organisms. One relatively rare, but intriguing, type of reorganization event involves the loss of DNA sequences by DNA breakage and rejoining events. In the copepod *Cyclops*, segments of heterochromatin are eliminated from internal regions of the chromosomes and the remaining segments rejoined (1). The significance of this particular process is unknown. However, in other organisms, DNA breakage and rejoining is required for the correct developmental assembly of protein coding regions. Genes coding for both immunoglobulins and T-cell receptors are assembled from DNA segments during the development of the vertebrate immune system (2). In response to low nitrogen conditions, the cyanobacterium *Anabaena* utilizes DNA breakage and rejoining for the assembly of a gene involved in nitrogen fixation (3). Most recently, DNA breakage and rejoining has been shown to be required for the formation of a gene encoding a sporulation-specific transcription factor in *Bacillus subtilis* (4).

Many DNA breakage and rejoining events also appear to occur during macronuclear development in the hypotrichous ciliated protozoa. Following conjugation, these unicellular protozoa transform a germline micronucleus containing chromosomes into a vegetative macronucleus containing short, linear DNA molecules (reviewed in 5,6). At the cytological level, macronuclear development can be divided into three stages. During the first stage, the micronuclear chromosomes in the developing macronucleus (anlage) undergo multiple rounds of replication to form polytene chromosomes. The second stage involves encasement of chromosome fragments in vesicle structures. While the vesicles are present, extensive DNA elimination occurs. The vesicles break down during the third and final stage of macronuclear development, and the remaining short, linear DNA molecules undergo additional rounds of DNA replication resulting in the mature macronucleus. The entire process, from mating to the appearance of the new macronucleus, occurs over a period of approximately 100 hr.

Comparisons of cloned macronuclear DNA molecules and their micronuclear precursors have shown that sequences are not only removed from between the precursors of macronuclear DNA molecules, but also from within them (7–10). These sequences, which have been termed internal eliminated sequences (IESs), are excised and the flanking DNA sequences are rejoined. IESs range in size from 14 bp to at least 500 bp and it has been estimated that tens of thousands of IESs are eliminated during macronuclear development in both *Oxytricha nova* (8) and *Euplotes crassus* (11). IESs differ in their primary sequences and appear to represent part of the unique DNA eliminated during development. Different IESs do, however, display two structural similarities. Each IES is bounded by a 2–6 bp direct repeat; one copy of the direct repeat remains in the mature macronuclear DNA molecule following developmental removal of the IES. Many IESs also have short, often imperfect, inverted repeats near their termini. Some IESs interrupt coding regions (10,12,7), so that their correct developmental removal is required for proper gene function. The IES phenomena is not limited to hypotrichous ciliates, as similar events have been documented in the holotrichous ciliate *Tetrahymena thermophila* (e.g., 13–15). There are fewer developmental DNA deletion events in *Tetrahymena* (~7,000), but the segments of eliminated DNA are generally larger.

More recently, two additional classes of sequences, which may be related to IESs, have also been found to be eliminated from internal regions of the micronuclear chromosomes. First, large, repetitive, transposonlike elements have been found to be developmentally excised from the precursors of macronuclear DNA molecules in both *E. crassus* (11,16) and *Oxytricha fallax* (17). Second, Greslin et al. (10) have found that the micronuclear copy of the actin gene of *O. nova* contains numerous interruptions and the coding sequences are scrambled. The macronuclear actin

gene must be assembled during development in concert with the removal of the interrupting sequences.

A number of models have been proposed for the mechanism of IES removal (18,8,15). In an attempt to distinguish between these models, we have examined the developmental timing of IES removal in *E. crassus*. DNA has been isolated from macronuclear anlagen at various times of development and IES removal assessed by Southern hybridization. Additional analyses were performed to correlate the time of IES removal with the cytological stage of macronuclear development. The results indicate that IES removal is an early event in macronuclear development, occurring late in the polytene chromosome stage, but preceding the chromosome fragmentation events that generate free macronuclear DNA molecules. The results are discussed in relation to the various models of IES removal.

MATERIALS AND METHODS

Culture and Mating of *Euplotes crassus*

Cells were grown as described by Roth et al. (19), except that vitamin B12 was omitted from the artificial sea water. The alga *Dunaliella salina* was used as the food source. Unless otherwise noted, matings were carried out with *E. crassus* strains CC51 (mating type III; mt^3/mt^1) and CC55 (mating type I; mt^1/mt^1) at room temperature. The mating system has been described by Heckmann (20).

For large scale matings, 10 l of each cell line were grown. For this purpose, a dense alga culture was filtered through 8 μm Nitex (Tetko, Inc., Lancaster, NY) and distributed to 2.8 l Fernbach flasks (2 l/flask). Penicillin and streptomycin were added to a concentration 100 U/ml and 100 μg/ml, respectively, to block bacterial growth. Each flask was inoculated with 100−200 ml of a dense *E. crassus* culture and grown at room temperature until the algal food source was depleted. After an additional 2 to 3 days of starvation, mating was initiated by mixing equal volumes of each cell line in 16″ × 10 3/4″ × 3 3/4″ plastic sweater boxes (2−4 1/box).

Isolation of DNA from Cells Undergoing Macronuclear Development

Macronuclear anlagen were isolated based on a procedure originally described by Roth et al. (19). At the indicated times, mated cells were filtered through 35 μm Nitex and collected on 15 μm Nitex. Cells were then harvested by centrifugation at 100×g for 1 min and the supernatant removed. The cell pellet was resuspended in 4.5 ml of room temperature 10 mM Tris (pH 7.5), 0.05% spermidine phosphate. Triton X-100 was added to 0.5%, the mixture quickly transferred to a siliconized Dounce homogenizer and the cells broken with 8−10 strokes. Subsequent steps were performed at 4°C. The lysed cell suspension was filtered through 25 μm Nitex, which allowed the passage of free nuclei including anlagen, but retained unlysed cells. One-fifth volume of Percoll solution (10 mM Tris, pH 7.5, 5% sucrose, 0.5% Triton X-100, 0.05% spermidine phosphate in Percoll) was added to the filtrate. The filtrate was then layered on top of a 24 ml 25−50% Percoll solution step gradient (4 ml steps in 5% increments). The gradient steps were made by diluting Percoll solution with '5% sucrose solution' (10 mM Tris, pH 7.5, 5% sucrose, 0.05% spermidine phosphate). The gradients were subjected to centrifugation at 1,000×g for 15 min. Visible bands, which formed at the step interfaces, were removed and examined under the microscope for the presence of anlagen. In most cases, the fraction(s) containing anlagen was diluted with 1/4 volume 5% sucrose solution and rerun over a smaller (12 ml) Percoll step gradient prepared as above.

The final anlagen fraction(s) was diluted with 6 volumes of 5% sucrose solution and the anlagen collected by centrifugation for 10 min at 1,000×g. The resulting pellet was resuspended in 500 ml of *Euplotes* cell lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 250 mM NaCl, 0.5% SDS) and Proteinase K was added to a final concentration of 200 μg/ml. The solution was then incubated at 37°C for 2 hr, after which the DNA was further purified on a CsCl gradient as described by Roth et al. (19).

Total cellular DNA (whole cell DNA) was isolated from aliquots of cells during a single large scale mating. At 5 hr intervals, 2 l of cells were harvested as described above. The cell pellets were stored at −70°C until DNA was isolated essentially as described by Klobutcher et al. (21).

Molecular Biological Techniques

DNA was digested with restriction enzymes under the conditions described by the manufacturer (New England Biolabs, Beverly, Mass. or Life Technologies, Inc., Gaithersburg, MD). DNAs were sized by electrophoresis through agarose or low-melting-point (LMP) agarose gels (Life Technologies, Inc.) prepared and run in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA , pH 8.0). The 1 Kb DNA Ladder (Life Technologies, Inc.) was generally used for size standards.

Southern blots were prepared using either nitrocellulose or Gene Screen Plus (New England Nuclear) as described by Southern (22). When Gene Screen Plus was used, the transfer buffer was 10× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0). Hybridization conditions were as described by Klobutcher et al., (23) except that the hybridization buffer for Gene Screen Plus was 6× SSC, 0.7% SDS, 1× Denhardt's solution (24). Hybridization probes were labeled with ^{32}P according to the random oligonucleotide priming method of Feinberg and Vogelstein (25,26). Following hybridization, filters received two 30 min washes with 2× SSC, 0.5% SDS followed by two 30 min washes with 0.1× SSC, 0.5% SDS at 65°C, unless otherwise noted. Autoradiograms from Southern blots were scanned using a Bio-Rad (Richmond, CA) 650 video densitometer and 1-D analyst software. The data presented represents the average of five scans.

Bacterial plasmid DNAs were prepared using the alkaline-lysis procedure (24). Recombinant bacteriophage were purified on glycerol step gradients and DNA was prepared as described by Maniatis et al. (24).

Preparation of Fixed Cells and Nuclei

During the course of a large scale mating, samples of fixed cells and nuclei were prepared at 2.5 hr intervals. For preparation of fixed cells, 15 ml of cells were pipetted from the bottom of a mated cell culture and the cells collected by centrifugation at 100×g for 1 min at room temperature. The supernatant was removed and 10 ml of room temperature 10 mM Tris-Cl (7.5), 0.25 M NaCl, 2 mM $CaCl_2$, 0.1% Triton X-100 was added to the cell pellet. The tube was inverted gently 5 times and the cells collected by centrifugation as above. The supernatant was aspirated, the cell pellet resuspended in 750 μl of ice cold methanol/acetic acid (3:1 or 9:1), and stored at 4°C. This procedure breaks the cell pellicle and releases much of the cytoplasm.

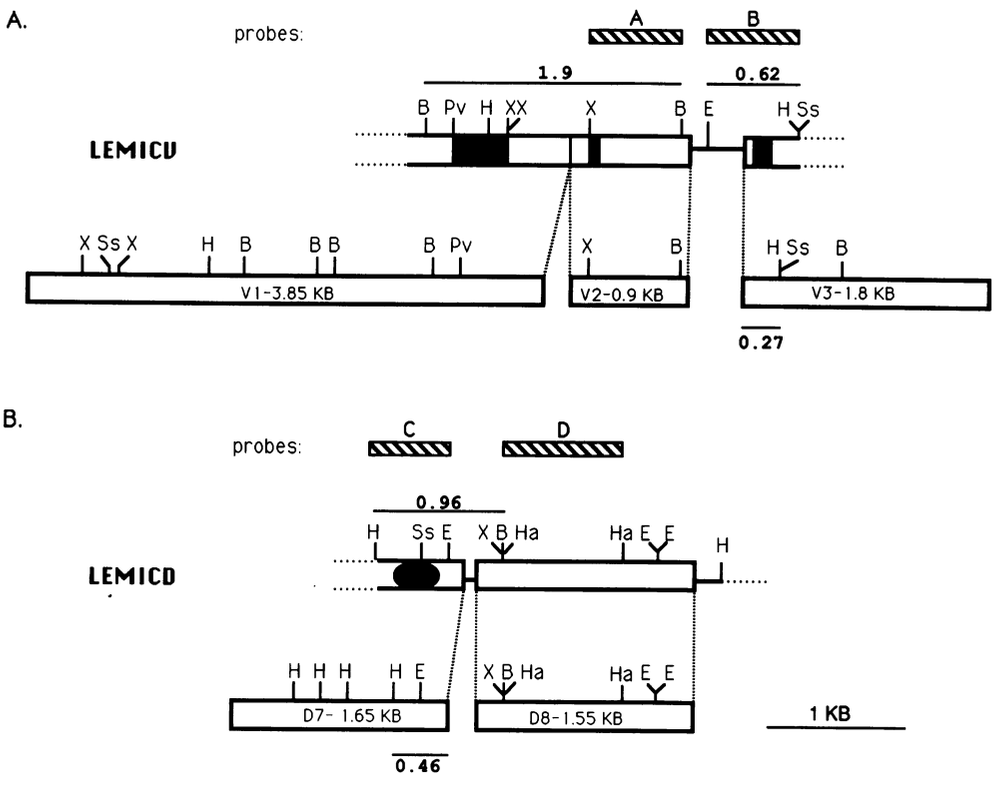

Figure 1. Maps of the Regions of the Micronuclear Genome Analyzed. A.) Restriction maps of a portion of micronuclear clone LEMICV and the three macronuclear DNA molecules (V1, V2, and V3) derived from this region during development. In the map of LEMICV, the areas which give rise to macronuclear DNA molecules are represented by open boxes, spacers separating adjacent macronuclear precursors are shown as lines, and IESs are indicated by black boxes when their sequence is known and by black ovals when it is not. Sizes of selected restriction fragments referred to in the text are indicated in kilobase pairs. DNA fragments used as hybridization probes are shown as hatched boxes. B.) Restriction maps of a portion of micronuclear clone LEMICD and the two macronuclear DNA molecules (D7 and D8) derived from this region. Other aspects are as described above. B = Bgl II, E = Eco RI, H = Hind III, Ha = Hae III (not all sites are shown), Pv = Pvu II, Ss = Sst I, and X = Xba I. All hybridization probes were derived from the micronuclear clones (or subclones) and represent the following fragments: probe A, 715 bp Xba I-Bgl II; probe B, 625 bp Eco RI-Hind III; probe C, 500 bp Eco RI-Hind III; probe D, 750 bp Hae III.

The fluorescent dye 4′-6-diamidino-2-phenylindole (DAPI) was used to stain fixed cells for microscopy. A 2 μg/ml solution of DAPI was prepared in 10 mM Tris-Cl, 1 mM EDTA (pH 7.5) to which β-mercaptoethanol was added to 1% to deter fading (27). Five to ten μl of DAPI were added to an equal volume of fixed cells and 10 μl of this mixture was placed onto a glass slide and immediately covered with a glass coverslip.

Fixed nuclei were prepared as described above, except that the initial cell pellet was resuspended in low ionic strength lysis buffer (10 mM Tris-Cl, pH 7.5, 0.5% spermidine phosphate, 0.1% Triton X-100). This procedure released the nuclei from the cells. For staining, 10 μl of fixed nuclei were placed on a glass slide and allowed to air dry. Six μl of the 2 μg/ml DAPI solution were then applied to the slide and a coverslip mounted.

Stained fixed cells and nuclei were examined on a Nikon Diaphot-TMD microscope equipped with epi-fluorescence. Photographs were taken with an attached Nikon FE2 35 mm camera using Kodak Tri-X Pan black and white film.

Quantitative Analysis of Anlagen DNA Content

Slides of DAPI-stained fixed cells were observed under oil with a 25× neofluor objective on a Zeiss IM35 fluorescent microscope. To determine the fluorescence intensity of micronuclei and macronuclear anlagen, frozen video images of cells were taken using a Hamamatsu camera linked to the ARGUS 100 VIM image analysis system (version 3.0, Hamamatsu Photonics, Oakbrook, IL). The area of the cell containing the desired nucleus was selected and the total fluorescence intensity in this region was measured using the ARGUS system. An equal area of the cell lacking nuclei was used to obtain a background reading which was subtracted from the nuclear fluorescence value.

To test if the micronuclear DNA content remained constant during anlagen development, the fluorescence intensity ratios of micronuclei from developing cells to micronuclei from starved, vegetative cells were determined. Before fixation, cells at specific developmental times were mixed 1 to 1 with vegetative cells. Microscopic fields containing both a vegetative and a developing cell were frozen and the fluorescence intensity of a micronucleus from each cell was determined. For each developmental time, a minimum of 15 vegetative and developing cell pairs were examined.

To analyze changes in the DNA content of developing macronuclei, cells containing well-separated anlagen and micronuclei were selected and the fluorescence intensity of each nucleus was determined. The ratio of anlagen to micronuclear fluorescence was calculated for a minimum of 20 cells at each developmental time. Using this approach, one occasionally misidentifies a small fragment of the old, degenerating macronucleus as a micronucleus, resulting in a highly aberrant anlage/micronuclear fluorescence ratio. As such values greatly affected the mean value of the population, the following policy was adopted. First, the mean and standard deviation of the

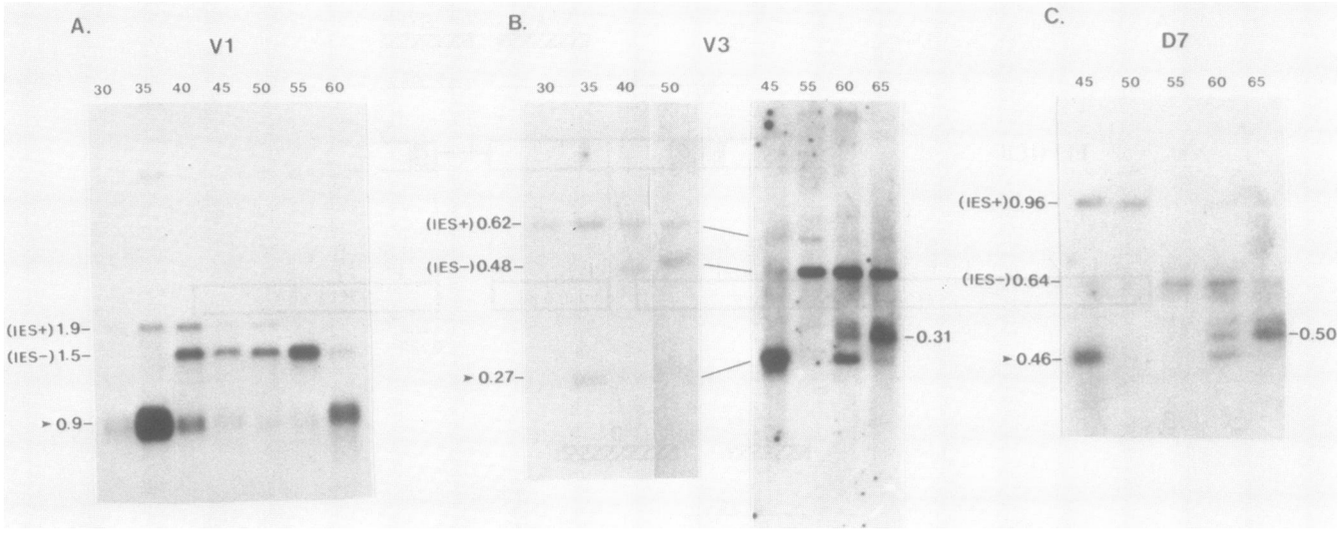

Figure 2. Southern Hybridization Analyses to Detect Removal of the V1, V3, and D7 IESs during Macronuclear Development. Southern blots containing restriction digests of approximately 1 μg of anlagen DNA from cells 30–65 hr post-mixing (lanes 30–65) were hybridized with radiolabeled probes indicated in Figure 1. Hybridizing fragments are indicated in kilobase pairs. 'IES+' and 'IES–' denote expected sizes of fragments containing and lacking the IES, respectively. An arrowhead denotes the expected size of contaminating mature macronuclear DNA fragments homologous to each probe. A.) Hybridization of probe A to Bgl II digested anlagen DNA preparations. B.) Hybridization of probe B to Eco RI + Hind III digested anlagen DNAs. Final washes of the filter were in 0.5× SSC, 0.5% SDS at 65°C. C) Hybridization of probe C to Hind III + Xba I digested anlagen DNAs. Final washes of the filter were in 0.5× SSC, 0.5% SDS at 65°C.

anlage/micronucleus fluorescence intensities for the total population of cells were determined. Values that differed from the mean by more than two standard deviations were eliminated and the mean and standard error were then recalculated. Overall, less than 5% of the cells examined were excluded.

RESULTS

Timing of IES Removal

The developmental removal of IESs was examined in two regions of the micronuclear genome that previously had been cloned and characterized (11). The first region, represented within clone LEMICV (Fig. 1A), contains the right end of the precursor of a macronuclear DNA molecule referred to as V1, the entire V2 precursor, and the left end of the V3 precursor. Within this region, IESs of 374, 31, and 144 bp are present in the V1, V2, and V3 precursors, respectively. The second region of the micronuclear genome analyzed, represented by clone LEMICD (Fig. 1B), gives rise to the right end of D7 as well as the entire D8 macronuclear molecule. Hybridization analysis has indicated that one or more IESs totalling 330 bp reside near the right end of the D7 precursor (11).

To determine the developmental timing of removal of the V1 IES, anlagen were isolated from cells at 5 hour intervals between 30 and 65 hr after mixing starved cultures of the *E. crassus* cell lines CC51 and CC55 (all developmental times refer to hours post-mixing). The DNAs obtained from the anlagen preparations were digested with Bgl II, size fractionated on an agarose gel, Southern blotted, and hybridized with probe A (Fig. 1A). At early times, 30 to 40 hr post-mixing, hybridization to 1.9 kbp fragments was observed (Fig. 2A). This is the expected size of the Bgl II fragment containing the V1 IES (IES+ form) in unrearranged micronuclear or unrearranged anlagen DNA (Fig. 1A). Beginning at 40 hr, hybridization to 1.5 kbp fragments is observed and this becomes the major hybridizing size class at later times. A 1.5 kbp Bgl II fragment (IES–form) is the size one would predict if the 374 bp VI IES was removed from the V1 precursor. Note that removal of the 31 bp V2 IES, also contained in the 1.9 kbp Bgl II fragment, could not be detected in this analysis because of its small size.

Hybridization to 0.9 kbp fragments was also evident in some lanes (Fig. 2A). The mature V2 macronuclear DNA molecule, which shares homology with probe A, is 0.9 kbp in size (Fig. 1A). Thus, the 0.9 kbp band is likely due to variable levels of contamination of the anlagen preparations with fragments of the old, vegetative macronucleus, which persist in the cell until late in macronuclear development (19,28; see Fig. 5). The mature vegetative macronucleus has approximately 1000 copies of each macronuclear DNA molecule (29) so that even a small amount of contaminating vegetative macronuclei can produce a strong signal on a Southern blot.

The removal of the 144 bp V3 IES and the 330 bp D7 IES(s) were examined using similar strategies. For the V3 IES, only IES+ forms were detected at 30 and 35 hr post-mixing (Fig. 2B). Beginning at 40 hr, fragments corresponding to the predicted IES– form appeared, and became the predominant hybridizing form by 55 hr. Additional hybridizations were done using a probe specific for the micronuclear DNA separating the V2 and V3 precursors to confirm that the putative IES– band represented removal of the V3 IES and not the removal of the intergenic spacer. The intergenic spacer probe hybridized to both the IES+ and IES– forms (data not shown), indicating that the smaller fragments lack the V3 IES and that the intergenic spacer has not yet been eliminated. For the 330 bp D7 IES(s), IES– forms first appeared at 55 hr, and constituted the major hybridizing species at this time (Fig. 2C).

The above analyses also provided an indication of the time of excision of macronuclear DNA molecules from the chromosome. Roth and Prescott (30) previously have found that free macronuclear DNA molecules first appear during development with an extra 40 bp of telomeric repeat sequences (hypotrich telomeres consist of repeats of the octanucleotide 5′CCCCAAAA3′, or 'C_4A_4 repeats') at each end relative to the mature macronuclear DNA molecule. Evidence for 'extended

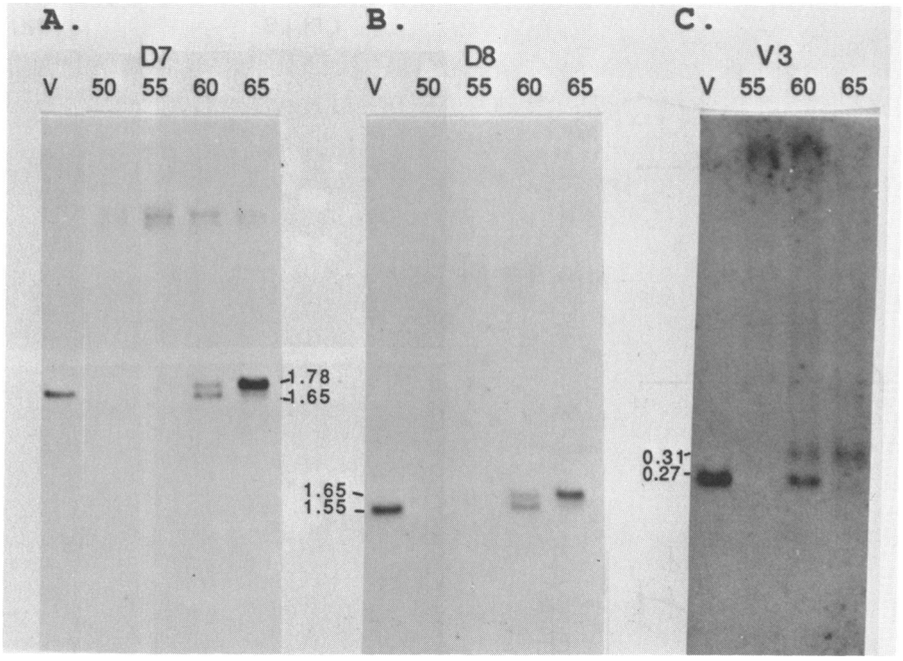

Figure 3. Appearance of Extended Telomere Forms of the D7, D8, and V3 Macronuclear DNA Molecules. Hybridization of D7, D8, and V3 specific probes (see Fig. 1) to Southern blots containing DNA isolated from anlagen 50 to 65 hr after cell mixing (lanes 50−65) and to whole cell DNA isolated from vegetative cells (lane V). Vegetative cell DNA was included on the gel to show the size of the mature macronuclear DNA molecule. A.) Hybridization of D7-specific probe C to undigested anlagen and vegetative cell DNAs. Final washes were in 1× SSC, 0.5% SDS at 65°C. B.) Hybridization of D8-specific probe D to undigested anlagen and vegetative cell DNAs. C.) Hybridization of V3-specific probe B to Hind III digested anlagen and vegetative cell DNAs. This strategy detects left terminus-Hind III restriction fragments of both the mature macronuclear and the extended telomere form of V3. Final washes were in 0.5× SSC, 0.5% SDS at 65°C. In each case, sizes of hybridizing molecules are indicated in kilobase pairs. Note that the hybridization to mature macronuclear forms in 60 hr anlagen DNA is likely the result of contamination of this preparation with DNA from the old macronucleus.

telomere' forms was observed in the hybridizations designed to detect IES removal. At 60 and 65 hr, hybridization to 0.31 and 0.50 fragments was observed in the V3 and D7 hybridization experiments, respectively (Fig. 2B, C). These fragments are approximately 40 bp larger than the expected sizes for terminal restriction fragments of V3 and D7 (Fig. 1), and represent extended telomere forms. In the hybridization to detect V1 IES removal (Fig. 2A), the signal at ~0.9 kb at 60 hr also likely represents hybridization to extended telomere forms of V2, but the gel was not capable of unambiguously resolving extended telomere forms from mature macronuclear DNA molecules.

A series of additional hybridizations to anlagen DNAs were performed to systematically examine the appearance of extended telomere forms or other subchromosomal DNA intermediates. For D7 and D8, Southern blots containing undigested anlagen DNAs were prepared and hybridized with the D7 and D8 specific probes C and D (Fig. 1B), respectively. Through 55 hr of development, no extended telomere forms or other low molecular weight DNA intermediates were detected (Fig. 3A, B). Beginning at 60 hr, hybridization to molecules ~100 bp larger than the mature macronuclear D7 and D8 molecules was observed, indicating the appearance of the free extended telomere forms. Free, extended telomere forms of V1, V2, and V3 were also first observed at 60 hr post-mixing (Fig. 3C; data not shown).

Two aspects of the above results demonstrate that IES removal occurs early in macronuclear development, preceding chromosome fragmentation. First, in the experiments examining V1 and D7 IES removal, the fragments detected in the Southern hybridizations span the termini of two precursors of macronuclear DNA molecules (Fig. 1), and thus two developmental fragmentation sites. The ability to detect fragments whose sizes are consistent with the micronuclear configuration minus the IESs indicates that neither of the fragmentation events required to generate the ends of the two macronuclear DNA molecules has yet occurred. Second, IES removal is observed prior to the appearance of extended telomere forms. With one exception, Roth and Prescott (30) found that the first appearance during development of subchromosomal species of macronuclear DNA molecules is as an extended telomere form.

IES Excision Occurs during the Polytene Chromosome Stage

In the above hybridizations with anlagen DNAs, there was some apparent variation in the time of removal for the three IESs. Removal of the V1 IES and V3 IES began at 40 hr, but IES− forms of D7 were not observed until 55 hr (Fig. 2). Although this may represent true variation in the time of IES removal, it is also possible that the time differences arose from experimental variability in the rate of macronuclear development. The DNAs representing each time of development were isolated from independent matings, and different anlagen DNA preparations were sometimes used on different blots. This was necessary because large numbers of cells were required to isolate significant amounts of anlagen DNA. To determine if the various IESs were actually removed at different times, a single large mating was performed and samples of whole cell DNA were prepared at 5 hr intervals for Southern analysis. Samples of fixed cells and nuclei were also prepared from this mating at 2.5 hr intervals to determine whether IES removal occurs during the polytene chromosome stage or the vesicle stage of development.

Cell lines CC51 and CC55 were used in the large mating, and

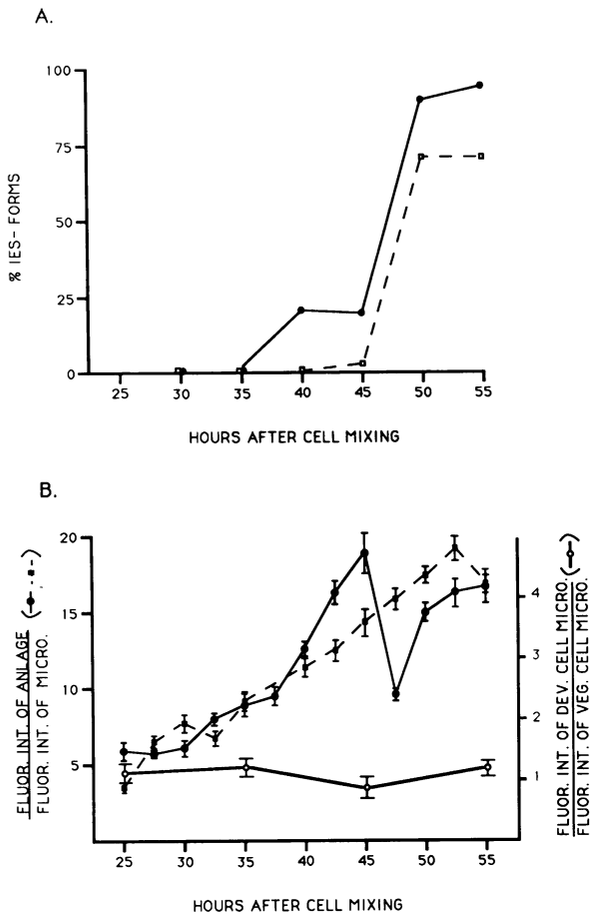

Figure 4. IES Removal and Increase in DNA Content of the Anlagen during the Polytene Chromosome Stage. A.) The percent IES− forms for the 374 bp V1 IES (filled circles) and the 330 bp D7 IES (open squares) are plotted versus time (hr) after cell mixing. The percent IES− forms, relative to the total of IES+ and IES− forms, was determined by densitometric scanning of autoradiographs from Southern hybridizations using whole cell DNA preparations from the mating time course. B.) The ratio of the fluorescence intensity of the anlage to the fluorescence intensity of the micronucleus as a function of time after cell mixing is shown. Fixed cells from the mating time courses were stained with DAPI and the fluorescence intensity of nuclei determined as described in Materials and Methods. Values from the same mating time course used to quantitate IES+ and IES− forms are shown (filled circles) as well as values from a second independent mating involving cell lines 14 and CC100 (filled squares). A minimum of 20 cells were examined at each developmental time. Also shown is the ratio of fluorescence intensities of the micronucleus in developing cells to the micronucleus in starved vegetative cells (open circles) as a function of developmental time. Values shown represent the mean ± the standard error of the mean.

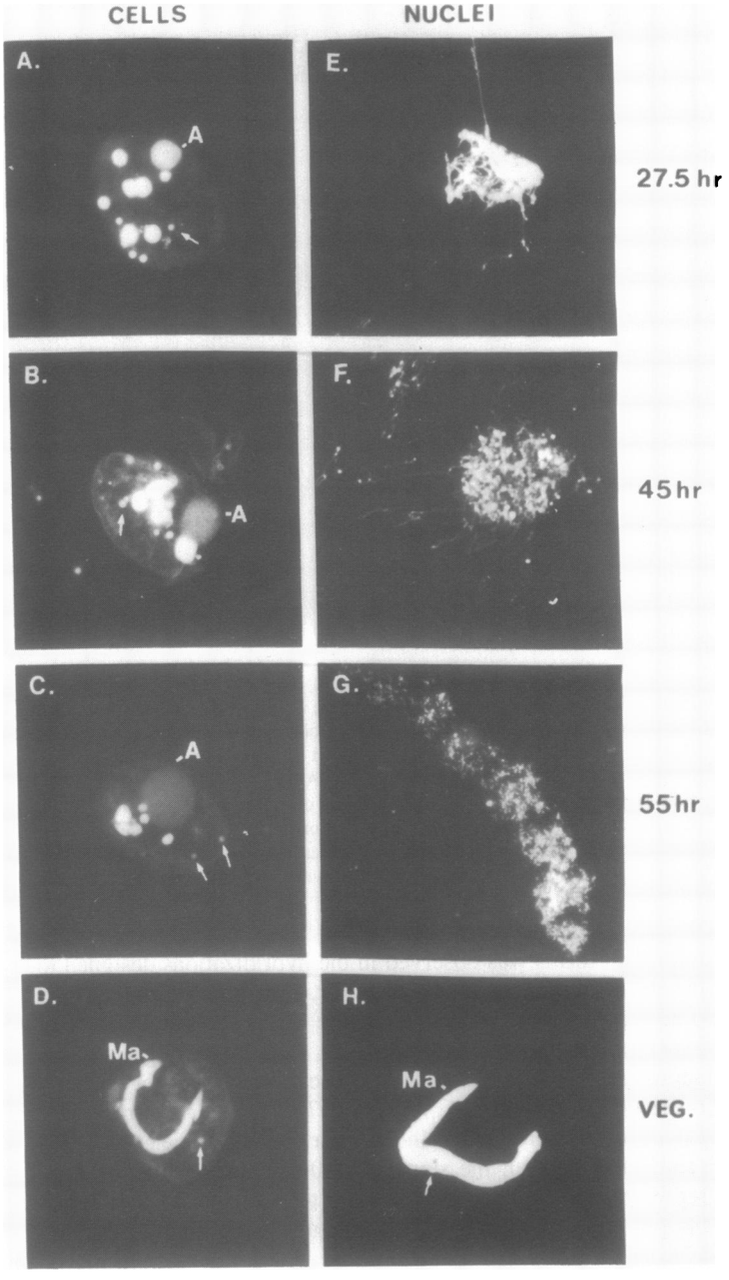

Figure 5. Cytology of Cells Undergoing Macronuclear Development. Preparations of *E. crassus* fixed cells and nuclei were made and stained with DAPI as described in Materials and Methods. Fixed cells from 27.5 hr post-mixing (A), 45 hr (B), and 55 hr (C) are shown along with a vegetative cell (D). DAPI-stained nuclear spreads are from 27.5 hr post-mixing (E), 45 hr (F), and 55 hr (G). Panel H is a macronucleus and micronucleus from a vegetative cell. Micronuclei are indicated by arrows, anlagen by an 'A', and mature macronuclei by 'Ma'. Other brightly staining bodies in fixed cells represent fragments of the old macronucleus or meiotic products. Note that the procedure for preparing fixed cells partially ruptures the cell cortex, so that some nuclei and fragments are often lost. The cells shown were selected because they retain the anlage and at least one micronucleus. Photographs were taken at a magnification of 400×.

temperature was maintained at 24°C. Microscopic observation of cells indicated a relatively high degree of mating synchrony. Within four hours of mixing, approximately 70% of the cells had formed mating pairs. However, additional mating occurred later, as greater than 90% of the cells ultimately entered macronuclear development.

Removal of the V1 and D7 IESs were examined using the same hybridization strategies described in Figure 2, except that the areas of the blots that contained the mature macronuclear DNA molecules were cut off prior to hybridization. This step was added to avoid the strong hybridization produced by the mature macronuclear DNA molecules (from the old, degenerating macronucleus) in the whole cell DNA preparations. After hybridization and autoradiographic exposure of the blots, the relative amounts of hybridization to IES+ and IES− forms were determined by scanning densitometry. Figure 4A presents the percent hybridization to IES− forms, relative to the total hybridization to IES+ and IES− forms, at various times during development. A minor difference in the time of first appearance of the IES− forms was observed. V1 IES− forms were first

detected at 40 hr, while the D7 IES− forms begin to appear at 45 hr. However, there was a major conversion of IES+ to IES− forms from 45 to 50 hr for both IESs. In the case of V1, there is an increase from 20 to 90% IES− forms during this interval, while D7 IES− forms increase from 3 to 71%. After 50 hr, there appears to be no significant additional conversion to IES− forms. Note that complete conversion is not expected since the whole cell DNA contains some micronuclear DNA. The results indicate that although there may be some minor differences in the timing of the start of IES removal, the majority of the IESs are removed during a short interval.

To correlate the time of IES removal with the cytological stage of development, preparations of fixed cells and nuclei obtained 15−60 hr post-mixing were stained with the fluorescent DNA-binding dye 4′-6-diamidino-2-phenylindole (DAPI). In the fixed cell preparations, a lightly staining, small macronuclear anlagen was first unambiguously detectable at 25 hr post-mixing. Cells at this time also contained 1 or 2 micronuclei and numerous intensely staining fragments of the old, degenerating macronucleus (Fig. 5A). The size of the macronuclear anlagen increased over the next 35 hr (Fig. 5A−C). Analysis of DAPI stained nuclear spreads from 27.5 to 55 hr revealed thread-like structures representing polytene chromosomes (Fig. 5E,F). A large number of chromosomes was observed, as has been seen in other hypotrich species (see 5). At 55 hr, some nuclear spreads showed small, independent, dot-like staining, which signaled the entrance into the vesicle stage (Fig. 5G). By 60 hr, the majority of nuclear spreads displayed the vesicle pattern of staining. Based on these analyses, the major period of IES removal (45−50 hr) occurs near the end of the polytene chromosome stage.

Increase in Anlagen DNA Content during the Polytene Chromosome Stage

DAPI-stained fixed cells were also used to examine the increase in DNA content of the anlagen during the polytene chromosome stage using a video image analysis system (see Materials and Methods). Fluorescence intensity of DAPI-stained nuclei has been shown to be proportional to DNA content (31). In these analyses we wished to measure changes in anlagen DNA content relative to an internal standard to minimize any potential variability in cell-to-cell staining and/or cell preparations. The micronucleus in developing cells did not appear to increase in DNA content and was considered a candidate for such an internal standard. To determine if the DNA content of the micronucleus remains constant during development, we first compared the fluorescence intensity of DAPI-stained micronuclei in cells at various stages of development to micronuclei in starved vegetative cells. From 25 to 77 hr post mixing, the ratios of fluorescence intensities of the micronuclei in developing cells to micronuclei in vegetative cells did not vary significantly and remained close to 1 (Fig. 4B, open circles, and data not shown). This indicates that there is little change in micronuclear DNA content throughout this period of macronuclear development.

The ratios of anlagen fluorescence intensity to micronuclear fluorescence intensity were then determined for the staged series of cells undergoing macronuclear development (Fig. 4B, solid circles). At the first time examined, 25 hr post-mixing, the DNA content of the anlage was already approximately 6 times higher than that of the micronucleus. Over the next 20 hr, there was a steady increase in the DNA content of the anlage, reaching a value approximately 19 times that of the micronucleus at 45 hr. With the exception of a decrease in the DNA content to 9.6 times that of the micronucleus at 47.5 hr, the DNA content then leveled off at about 16 times that of the micronucleus up until the beginning of the vesicle stage. Assuming that the micronucleus has a DNA content of 2C, and that the entire genome replicates, the maximum level of polytenization would correspond to 32 to 38C. This is similar to the 30C value reported for the hypotrich *Stylonychia lemnae* at this developmental stage (32).

The decrease in DNA content between 45 and 47.5 hr is intriguing because it occurs during the period of maximum IES removal. It may represent the removal and degradation of IES sequences, which would then be followed by some additional DNA replication to return the anlagen DNA content to approximately 15 times that of the micronucleus (Fig. 4B). However, we are unsure of the significance of this observed decrease in DNA content, as it has not been consistently seen in repeated experiments (e. g., Fig. 4B, solid squares). What is clear from the data is that IES removal occurs late in the polytenization process and there is not a large net increase in the DNA content of the anlage during this period. Even allowing for the observed decrease in DNA content to 9.6 times that of the micronucleus, there is less than a doubling in the total DNA content of the anlage during the period of IES removal.

DISCUSSION

IES Removal and Chromosome Fragmentation Are Temporally Distinct Processes

The results presented indicate that IES removal is an early event in macronuclear development. Based on the data from the staged series of DNA preparations, the major period of IES removal occurs between 45 and 50 hr after cell mixing. Entry into the vesicle stage of macronuclear development, which correlates with the appearance of free extended telomere forms of macronuclear DNA molecules (19,30), does not occur until 55−60 hr after cell mixing. Thus, IES removal precedes the chromosome fragmentation events that generate free macronuclear DNA molecules. It has been argued previously that the DNA breakage-rejoining and chromosome fragmentation events during macronuclear development are related (33); chromosome fragmentation may simply be an alternative outcome of the DNA breakage step. This notion was supported by the observation that DNA breakage-rejoining and chromosome fragmentation occur at approximately the same time during macronuclear development in *T. thermophila* (reviewed in 15). Our results show a clear difference in the timing of the two processes. Although one can devise models by which temporally distinct events are mediated by the same cellular machinery, the data suggest that chromosome breakage and IES removal are different processes. The apparent discrepancy between the timing of the two classes of events in *T. thermophila* and *E. crassus* may simply be a matter of the different rates of macronuclear development in the two organisms. Macronuclear development in *E. crassus* occurs over a period of approximately 100 hr, while it requires only about 20 hr in *T. thermophila* (see 34) making it more difficult to resolve closely timed events. Alternatively, the DNA rearrangement processes in the two organisms may be dissimilar.

It should be noted that the time of appearance of the vesicle stage and extended telomere forms in our studies (~60 hr) is approximately 10 hr later than reported by Roth and co-workers (~ 50 hr; 19,30). This is likely the result of differences in the cell lines employed and specific laboratory conditions. In the future, comparisons of work from different laboratories will be

aided by molecular markers denoting particular sub-stages of development, such as the timing of IES removal reported here. Other useful molecular markers include the appearance of extended telomere forms of macronuclear DNA molecules (30), which occurs at the onset of the vesicle stage, and the excision of the Tec1 transposon-like element (16), which occurs early in the polytene chromosome stage.

Models of IES Removal

Our hybridization analyses suggest that there is a direct precursor-product relationship between the IES+ and IES− forms. That is, as IES− forms appear during development, the IES+ forms concomitantly disappear. This observation is most consistent with models of IES removal that require active intramolecular DNA breakage and rejoining. In related studies (35), we have observed free circular forms of IESs in developing cells beginning at the time IES− forms appear. The existence of such forms is again most easily explained by a intramolecular DNA breakage and rejoining process.

The data also argue against other possible models of IES removal (18,8,15). One possible model involves an intermolecular recombination event between the left direct repeat of an IES on one DNA molecule and the right direct repeat of an IES on another DNA molecule. Such unequal crossing over would be expected to generate one DNA molecule lacking an IES and another with a duplication of the IES. In the hybridization analyses, no evidence of molecules with multiple IESs was seen at the times when IES− forms appear.

A second model that has been proposed for IES removal involves transcription of macronuclear precursors, removal of IESs from the transcript by RNA splicing, and reverse transcription to form the macronuclear DNA molecule (18,8). Although this model cannot be ruled out entirely based on this study, two aspects of the data place additional constraints on the model. First, IES removal occurs while the DNA in the anlage is still of high molecular weight. This would require that both extremely large transcripts and reverse transcripts be made. Second, the concomitant disappearance of IES+ forms and the appearance of IES− forms would require that the DNA template be destroyed during, or immediately after, the transcription process.

Some aspects of the data also argue against a slipped pairing mechanism (36,37) for IES removal (8). If slipped pairing was exclusively responsible for IES removal, one round of DNA replication would generate heteroduplex DNA molecules with an IES on only one DNA strand. Subsequent rounds of replication would result in DNA molecules lacking IESs and the gradual dilution of heteroduplex forms. Heteroduplex forms might be expected to migrate differently from IES+ and IES− forms during gel electrophoresis, and again we saw no evidence for such forms in the hybridization experiments. In addition, the quantitation of DNA content of the anlage by DAPI-staining suggests that there is no more than a doubling of the net DNA content during the period of IES removal. Assuming this represents one round of DNA replication at most, it would not be sufficient to significantly dilute the heteroduplexes formed by one round of slipped pairing. There are, however, two potential means by which DNA replication could have been masked in our analysis. First, it is possible that both extensive DNA degradation and extensive replication are occurring in concert at the time of IES removal, resulting in the observed relatively stable DNA content. Second, there could be changes in the strucure of the chromatin during the time of IES removal that would affect the ability of DAPI to bind DNA. Given these caveats, we cannot completely rule out the possibility that extensive DNA replication is involved in IES removal. Nevertheless, the simplest interpretation of our data is consistent with work on *T. thermophila* (38), which clearly shows that no more than a single round of DNA replication occurs during the process equivalent to IES removal. Finally, there is a modified slipped pairing model that is still consistent with the data. A single round of slipped pairing might produce a heteroduplex and the IES remaining in one strand might then be removed by a DNA repair process. This would still involve active DNA breakage and rejoining, and does not alter our conclusion that IES removal is an active process occurring intramolecularly at the DNA level.

Additional studies will be required to provide details of the IES removal process. It will be of particular interest to try and reconstitute IES removal excision *in vitro*. Robinson et al. (39) have recently found that extracts derived from developing *T. thermophila* cells were capable of carrying out deletion-ligation reactions on a DNA substrate containing an IES from the hypotrich *O. nova*. Although correct excision of the IES was not observed, this may simply be a function of the interspecific nature of the *in vitro* system. Now that the timing of IES removal in *E. crassus* is known, it should be possible to prepare extracts from this organism and determine if they are capable of correctly catalyzing the excision of the organism's own IESs.

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