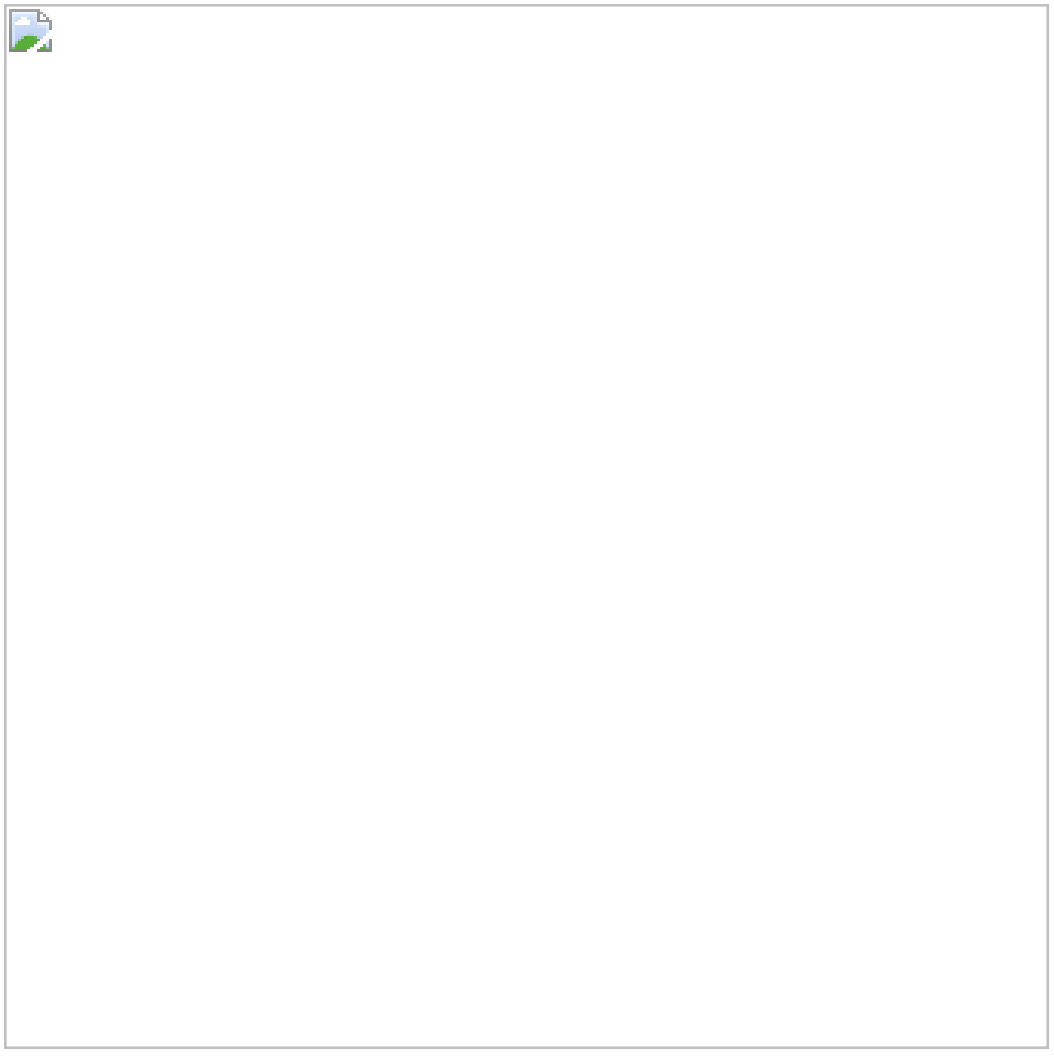

Partial Autolysis of Macronuclear Fragments Revealed by Rapid DNA Degradation in Exconjugants of Paramecium multimicronucleatum

Authors: Hirano, Yuko, Harumoto, Terue, Hara, Jyunko, and Takagi, Yoshiomi

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Partial Autolysis of Macronuclear Fragments Revealed by Rapid DNA Degradation in Exconjugants of *Paramecium multimicronucleatum*

Yuko Hirano, Terue Harumoto, Jyunko Hara
and Yoshiomi Takagi*

Department of Biological Science, Nara Women's University, Nara 630-8506, Japan

ABSTRACT—During autogamy or conjugation in *Paramecium*, the micronucleus undergoes prezygotic and postzygotic nuclear divisions to result in new micro- and macronuclei, while the old macronucleus breaks down into fragments. The fragments appear to remain functional for some time, as evidenced by their ability to generate an intact macronucleus, via the process called macronuclear regeneration. On the other hand, macronuclear DNA degradation must take place, as suggested by a transfer of isotope-labeled material from the fragments to the developing macronuclear anlagen. We monitored electrophoretic profiles of DNA in autogamous and exautogamous cells of *P. tetraurelia* at 1-day intervals for 5 days, and in conjugating and exconjugant cells of *P. multimicronucleatum* treated with conjugation-inducing chemicals at 1-2 hr intervals for 2 days. We failed to detect DNA degradation in *P. tetraurelia*, but found a prominent DNA electrophoretic smear in starving exconjugant cells of *P. multimicronucleatum* 33-40 hr after the initiation of conjugation. This DNA degradation was remarkable in that it occurred 10-20 hr after the appearance of macronuclear anlagen, in that it occurred suddenly and transiently, showing no trace one hour before and one hour after the smear was detected, and in that most of the macronuclear fragments persisted after the smear was detected. These results show that, during the starvation period in *P. multimicronucleatum* exconjugants, partial autolysis of macronuclear fragments occurs at a specific stage. We propose that this stage might be the critical point after which macronuclear fragments could not be regenerated to a functional macronucleus.

INTRODUCTION

During sexual reproduction of *Paramecium*, i.e., conjugation and autogamy, the germinal micronucleus undergoes meiosis and fertilization to produce a fertilized nucleus from which the new macro- and micronuclei differentiate, while the somatic macronucleus breaks down into fragments. The simultaneous occurrence of birth and death in the same cytoplasm suggests precise regulation between the two processes. Because the life-managing functions in ciliates are carried out by the macronucleus, including the initiation, commitment and prosecution of sexual reproduction (Mikami, 1979, 1988), macronuclear fragmentation must be a self-regulated, positive process somewhat like apoptosis in multicellular organisms. In fact, the inhibition of protein synthesis inhibits macronuclear fragmentation in *P. caudatum* (Mikami, 1996) and inhibits macronuclear condensation in *Tetrahymena thermophila* (Mpoke and Wolfe, 1996). In conjugating pairs of *T. thermophila*, it has been reported that apoptotic DNA degradation was detected in association with macronuclear condensation (Davis *et al*., 1992), or probably before the initiation of condensation (Mpoke and Wolfe, 1996), and that the DNA degradation was inhibited by inhibitors of gene expression such as actinomycin D and cycloheximide or by a nuclease inhibitor such as aurin (Mpoke and Wolfe, 1996).

In exconjugant cells of *P. tetraurelia*, both exponential decrease in the number and total volume of macronuclear fragments and exponential increase in the volume of macronuclear anlagen occurred simultaneously; during this period, lasting for several days under starvation conditions, the mean size of the surviving fragments remained unchanged, suggesting selective and individual autolysis of the fragments (Berger, 1974). Also, ^{3}H-thymidine residues in the macronuclear fragments were mostly incorporated in fully developed anlagen, suggesting DNA cleavage in macronuclear fragments into building blocks which are then utilized to synthesize DNA in developing anlagen (Berger, 1974). In exconjugant cells of *P. caudatum*, more than half of the macronuclear fragments persisted for at least a week under starvation, suggesting very slow autolysis, if any (Mikami, 1975, 1979). The nuclear process in *P. multimicronucleatum* is similar to that in *P. caudatum* (Takagi, Y., unpublished observations).

Macronuclear fragments should remain functional until a

* Corresponding author: Tel. +81-742-20-3422;
FAX. +81-742-20-3421.

certain stage, because they can be regenerated to form an intact macronucleus, in the process called macronuclear regeneration (MR) (Sonneborn, 1974), and because genes of the old macronucleus control the development of the new macronuclear anlagen (Harumoto, 1986; Duharcourt *et al.*, 1995).

As in *Tetrahymena*, apoptotic DNA degradation very likely occurs also in *Paramecium*. We anticipated that DNA degradation would be detectable at all times in starving exautogamous cells of *P. tetraurelia*, and at an early stage of macronuclear development in starving exconjugants of *P. multimicronucleatum*. However, we found that DNA degradation occurred suddenly and transiently in a late stage of conjugation in *P. multimicronucleatum*, and it was not detectable in exautogamous cells in *P. tetraurelia*. The implications of the sudden, transient DNA degradation in *P. multimicronucleatum* will be discussed.

MATERIALS AND METHODS

Cells

Stock 51 (mating type VII) of *Paramecium tetraurelia* and stocks of 49B (mating type IV) and CH312 (mating type IV) of *P. multimicronucleatum*, syngen 2 were used. To test for mating reactivity of the latter stocks, stock 53B (mating type III) was used as a tester.

Culture

The culture medium for *P. tetraurelia* was a 1% phosphate-buffered broth of 5 g/l Wheat-Grass-Powder (Pines Int., USA) inoculated with *Klebsiella pneumoniae* 2 days before use, and the culture medium for *P. multimicronucleatum* was Ca^{2+}-poor fresh-lettuce medium (Miyake, 1968a) inoculated with a non-pathogenic strain of *Klebsiella pneumoniae* 2 days before use.

Cells were cultured in flasks (200 ml, 500 ml, or 1000 ml) filled to 40% of the flask volume with culture medium. Cultures were handled on a clean bench and incubated at 25 ± 1°C.

Induction of autogamy

Autogamy of *P. tetraurelia* cells was induced by natural starvation. Single cells from a stock culture were isolated and grown first in depression slides and then in flasks. When cultures became transparent, indicating the shift from log to stationary phase as the result of food exhaustion, the day was designated as day 0. On the next day (day 1), sampled cells ($n \geq 100$) were monitored for autogamy by staining with Dippell's stain (Dippell, 1955). When all of the sampled cells contained macronuclear fragments, the culture was designated as a 100%-autogamous culture, and used for the present experiments. Cells for agarose gel electrophoresis were taken daily for 5 days. Cell aliquots of the ages of 4-5 fissions after the previous autogamy were used as autogamy-immature cells (Ishikawa *et al.*, 1998). Autogamy-immature culture was prepared by mixing 1 part of 100%-autogamous culture with 15 parts of culture medium, and allowing the mixed culture to divide about 4 times. The resulting culture was used at the stationary phase.

Chemical induction of conjugation

Conjugation of *Paramecium* can be induced chemically in a clone of the same mating type (Miyake, 1956, 1958). From the many effective methods available (for review, see Miyake, 1996), we chose the "KCl + acriflavine in Ca^{2+}-poor condition" method (Miyake, 1968b; Takagi, 1971).

Cells of the stationary-phase culture were checked for mating reactivity by mixing with tester cells, and highly mating-reactive cells were harvested by mild centrifugation. Nine combinations of 6, 8 and 10 mM KCl with 6, 8 and 10 μg/ml acriflavine (Nacalai) were tested in advance with a small cell sample in depression slides, and the most effective combination, determined by the ratio of conjugating pairs after 1 hr of the treatment, was used. Then, 50 ml of KCl-acriflavine mixture of the most effective combination, usually 8 mM KCl and 8 μg/ml acriflavine in final concentrations, was mixed with 50 ml of the suspension of cells ($4\text{-}6 \times 10^3$ cells/ml) in a petri dish. Even though the ratio of pair formation was less than 100%, the ratio of macronuclear fragmentation determined 1 day after the treatment was always 100% or very close to it, because this treatment induces autogamy in *P. multimicronucleatum* (Shimomura and Takagi, 1984). The cultures in which the ratio of macronuclear fragmentation was less than 90% were discarded.

Agarose gel electrophoresis

To prevent artificial DNA breakdown, we prepared DNA samples for electrophoresis without DNA extraction as follows.

The cell pellet ($8\text{-}9 \times 10^8$ cells/ml for *P. tetraurelia* and $4\text{-}6 \times 10^7$ cells/ml for *P. multimicronucleatum*) was treated with 20 μl of low-EDTA lysing solution (1% SDS, 50 mM EDTA, 10 mM Tris-HCl, pH 9.5) (Epstein and Forney, 1984) for 10 min at 65°C. Samples were then treated with 2 μl of 10 mg/ml RNase A (Sigma) for 60 min at 50°C, followed by treatment with 2 μl of 10 mg/ml proteinase K (Sigma) for 90 min at 50°C. Thirty μl of each sample was directly applied to a 2% agarose gel (Seakem, GTG) and separated at 100V for 30 min (Mupid-2, Advance). Since the amount of macronuclear DNA is more abundant than that of micronuclear and mitochondrial DNAs, the bulk of the DNA detected in agarose gels was considered to be macronuclear DNA. The DNA markers used were a ϕX174/*Hae*III digest (Toyobo) and a λ/*Hin*dIII digest (Toyobo). Gels were stained with ethidium bromide and photographed under a UV transilluminator (Atto, DT-20MP) using Tri-X pan 400 film (Kodak) with an R2 filter (Kenko). Photographs were scanned with an image scanner (GT-9500, Epson) and processed with the software Photoshop (Adobe).

Cytological observation of nuclear events

Samples were air-dried on slides, fixed in an ethanol-acetic acid mixture (3:1) for 20 min, and dried. After they were treated with 70% ethanol for 20 min and dried, they were stained with 10 mg/ml 4',6-diamidino-2-phenylindole (DAPI) and observed under a microscope equipped with an epifluorescent illuminator (Olympus, BH2-RFC). Fluorescent micrographs were taken with an automatic exposure controller (Olympus, PM-10A) loaded with Tri-X pan 400 film (Kodak).

RESULTS

Part 1. Autogamous cells of *P. tetraurelia*

As described in Materials and Methods, we collected samples of autogamous and exautogamous cells from day 1 to day 5, and samples of autogamy-immature cells. On day 1, all autogamous cells possessed macronuclear fragments, which decreased in number to a few or zero on day 5. DNAs of the macronuclear fragments, therefore, should have degraded during this period. And yet we detected no sign of DNA degradation as an electrophoretic profile in samples collected once a day (Fig. 1). Note that the bulk DNAs consist of those from the macronuclear fragments, from the macronuclear anlagen and from the micronuclei. We conducted five experiments using different 100%-autogamous cultures, with consistent results (data not shown).

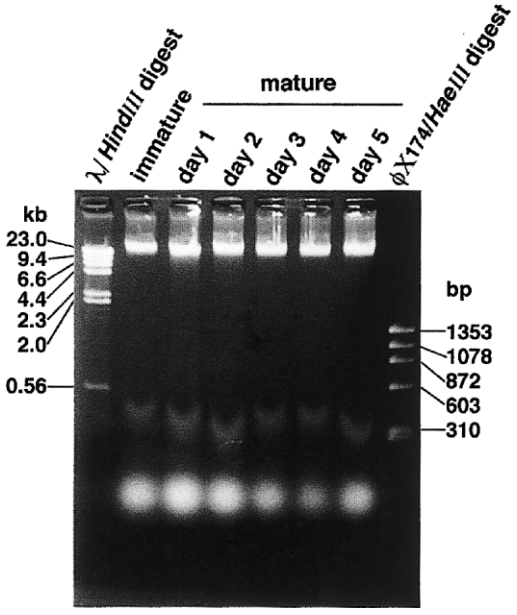

Fig. 1. Agarose gel electrophoresis of DNA in autogamy-immature and mature cells of *P. tetraurelia*. Autogamy-immature cells had undergone four fissions after autogamy. Mature cells of a 100%-autogamous culture induced by natural starvation were sampled daily for 5 days. A λ/*Hin*dIII digest and a φX174/*Hae*III digest are the DNA markers.

Part 2. Conjugating cells of *P. multimicronucleatum*

Search for DNA degradation in cells from 10 to 22 hr of conjugation

Chemically induced conjugants underwent well- synchronized nuclear events. After the onset of chemical induction, the macronucleus began to loosen at about 8 hr, and began to have a skein-form at about 10 hr. In cells of about 20-22 hr of conjugation, the old macronucleus was fully fragmented and the new macronuclear anlagen appeared. Thus, we first monitored DNA degradation on samples collected hourly from 10 to 22 hr after the onset of chemical induction. A sample collected at 30 hr was also monitored together with a control sample obtained just before the chemical treatment. The results of three experiments conducted on different cultures on different days are shown in Fig. 2 (A, B and C). No indication of a DNA ladder was detected. In these experiments, however, short, vague tailings of DNA were observed in most of the samples around 20 hr. However, these tailings consisted of DNAs with molecular weights larger than the marker of 1353 bp, and were thus not an indication of typical apoptotic DNAs, which should be ~200 bp and multiples thereof (Mpoke and Wolfe, 1996).

Search for DNA degradation in cells from 0 to 11 hr and those from 20 to 44 hr of conjugation

We monitored the samples collected from the periods missing in the above study, i.e., samples from 0 to 10 hr and from later than 22 hr. Cells were collected every hr from 0 to 11 hr and from 20 to 24 hr, and every 2 hr from 24 to 44 hr.

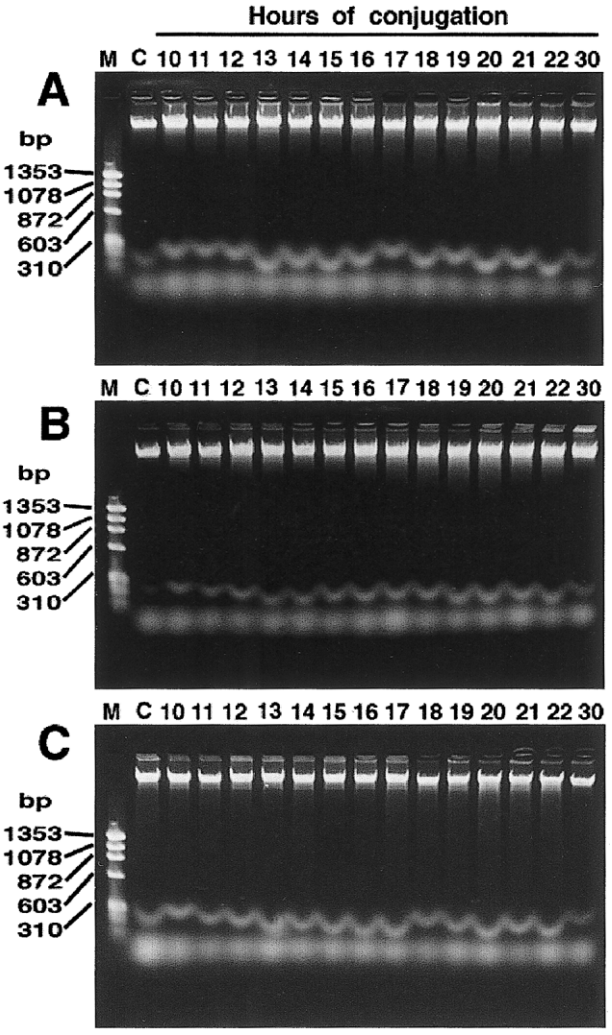

Fig. 2. Agarose gel electrophoresis of DNA in conjugating *P. multimicronucleatum* cells collected every hour from 10 to 22 hr and at 30 hr after the onset of chemical induction of conjugation. Three separate cultures (**A**, **B** and **C**) of stock CH312 were used. M; φX174/*Hae*III digest DNA marker. C; Control culture collected just before the chemical treatment.

The results of 2 separate experiments are shown in Fig. 3. In one of the experiments (Fig. 3A), a DNA smear reaching about 310 bp was detected in the sample collected after 40 hr of conjugation. No such smear was detected in samples collected 2 hr before and after that. Cells of 38, 40 and 42 hr of conjugation were stained with DAPI and observed with a fluorescence microscope (Fig. 4). Many macronuclear fragments and some macronuclear anlagen were seen in these three samples; however, no morphological characteristics specific to the 40-hr sample were detectable.

Search for DNA degradation in cells from 32 to 42 hr of conjugation

We then monitored samples collected hourly from 32 to 42 hr of conjugation. Among 15 chemically treated cultures, DNA smears were clearly detected in 3 cultures designated A, B and C (Fig. 5); Smears were detected in cells of 38 hr of

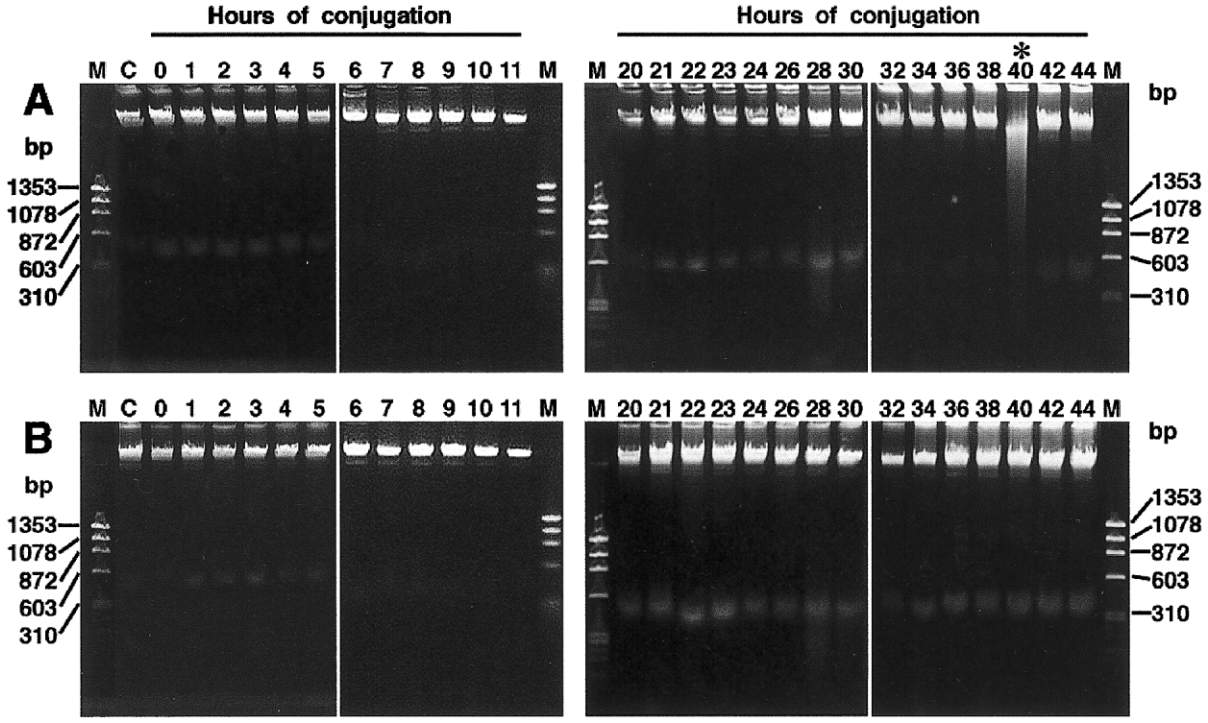

Fig. 3. Agarose gel electrophoresis of DNA in conjugating *P. multimicronucleatum* cells collected every hour from 0 to 11 hr and from 20 to 24 hr, and collected every 2 hr from 24 to 44 hr after the onset of chemical induction of conjugation. Two separate cultures (**A** and **B**) of stock CH312 were used. M; ϕX174/*Hae*III digest DNA marker. C; control culture collected just before the chemical treatment. Asterisk indicates a lane showing DNA degradation.

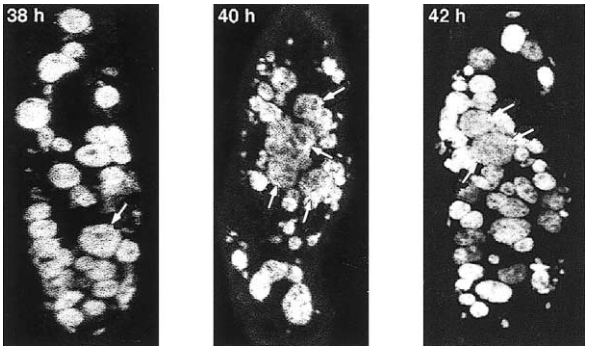

Fig. 4. Fluorescent micrographs stained with DAPI of *P. multimicronucleatum* cells collected 38, 40 or 42 hr after the onset of chemical induction of conjugation. The culture was the same as that used in Fig. 3A. One cell is shown for each hour of conjugation, because all samples were alike. Note that there were no morphological characteristics specific to the 40-hr sample, in which DNA degradation was detected in Fig. 3A. Arrows indicate macronuclear anlagen, and the other particles are macronuclear fragments. Although four macronuclear anlagen develop in *P. multimicronucleatum*, some were out of focus and therefore not visible.

conjugation in A, 33 hr in B, and 40, 41 and 42 hr in C. The smears detected in C were composed of DNAs larger than the largest marker 1353 bp. DNA degradation was not observed in the other 12 cultures.

DISCUSSION

During the starvation period in *P. tetraurelia* exconjugants, ^{3}H-labeled macronuclear fragments were individually and selectively autolysed, releasing soluble labeled material to yield precursors for the development of macronuclear anlagen (Berger, 1974). Since this process was reported to continue for 4-5 days after the end of conjugation, and nuclear events were reported to occur similarly in autogamy, we anticipated that DNAs in the macronuclear fragments would continuously degrade into fragments and thus DNA degradation would be detectable in agarose gel electrophoresis in all samples collected daily for 5 days from a 100%-autogamous culture. However, no DNA degradation was detectable (Fig. 1). It may be that the amount of DNA fragments was too small to be detectable as a smear because autolysis (possibly accompanying DNA degradation) of macronuclear fragments proceeded gradually. Alternatively, DNA degradation may occur stepwise and massively at one time, making its detection difficult in samples collected once a day from autogamous cultures induced by natural starvation, in which nuclear events would not proceed synchronously.

We therefore changed the species to *P. multimicronucleatum*, in which more synchronized nuclear processes were expected because of chemical induction of conjugation, and conducted surveys of DNA degradation in samples collected at intervals of 1-2 hr. We thereby succeeded in obtaining clear evidence of DNA degradation in samples collected 33, 38 and

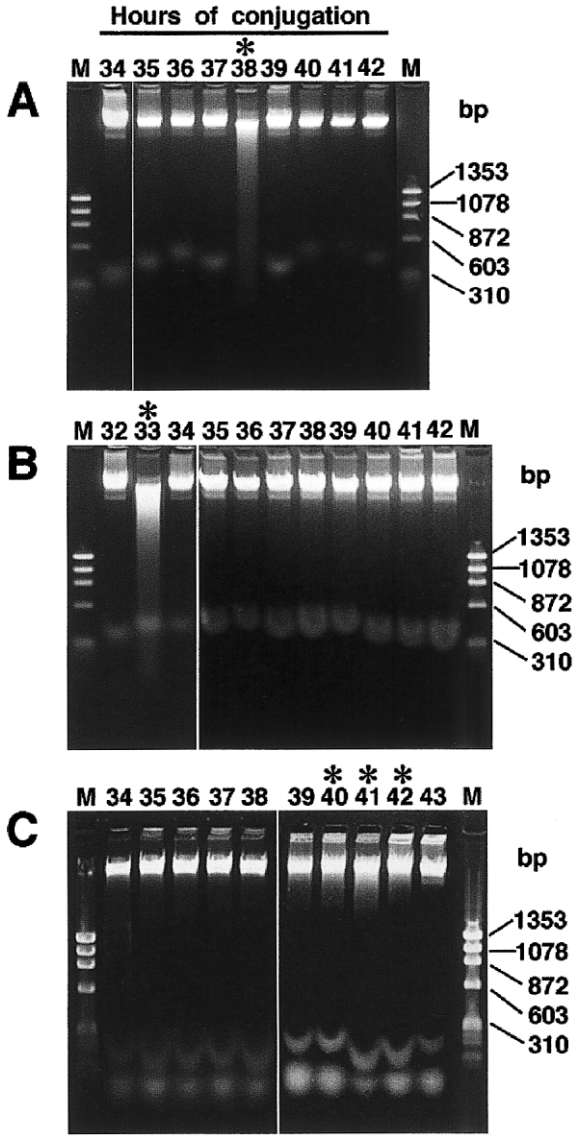

Fig. 5. Agarose gel electrophoresis of DNA in conjugating *P. multimicronucleatum* cells collected every hour after the onset of chemical induction of conjugation. (**A**) Cells of stock 49B were collected from 34 to 42 hr of conjugation. (**B**) Cells of stock 49B were collected from 32 to 42 hr of conjugation. (**C**) Cells of stock CH312 were collected from 34 to 43 hr of conjugation. M; ϕX174/*Hae*III digest DNA marker. C; Control culture collected just before the chemical treatment. Asterisk indicates a lane showing DNA degradation.

40 hr after the onset of chemical induction (Figs. 3, 5). The DNA electrophoretic profile was a smear, indicative of DNA degradation with products of random sizes, not a ladder like those indicative of DNA degradation at nucleosomal units. Small bands of about 300 to 350 bp seen in most of the electrophoresis gels consisted of DNA, because they were eliminated completely by DNase treatment (data not shown). These bands were not associated with DNA degradation, because they were also detected in the control samples. They might be a DNA reservoir stored somewhere in the cytoplasm.

In all of the 3 lanes in which long DNA smears were detected (Fig. 3A, Fig. 5A, B), the bulk DNA shifted a little bit downward and recovered to the original position in the next lane. This may indicate a rapid DNA degradation in macronuclear fragments, which may be followed by a rapid DNA synthesis in macronuclear anlagen utilizing degraded DNA as building blocks. Since the downshifted bulk DNA includes the DNA of the macronuclear anlagen as well, the DNA of the macronuclear anlagen was not of its final size at that time, but developed to (almost) the final size after 1 hr. The possibility that these smears are an artifact arising from inadequate preparation of the samples can not be completely ruled out. If it were the case, however, similar smears would appear more randomly and frequently; the long smears were detected only in 3 of 27 cultures studied (9 cultures were shown in Results), and only in 1 lane of each of 3 cultures representing 33-42 hr of conjugation.

It is well known that the micronuclear DNA sequences in ciliates are generally reorganized into the developing macronucleus, eliminating parts of the micronuclear DNA sequences (for review, Karrer, 1986; Klobutcher and Prescott, 1986). It is therefore possible that the partially eliminated sequences were detected as smears. This, however, should occur in early stages of macronuclear development. Because macronuclear anlagen appeared in 20-22 hr after the onset of chemical induction, the smears detected around this time (Fig. 2A-C, Fig. 3B) may be derived from the eliminated sequences.

Clear smears appeared in single lanes, but not in the neighboring lanes containing DNAs sampled at times differing by 1 or 2 hr (Fig. 3A, Fig. 5A, B). This indicates that DNA degradation occurred within a 1-hr time span and that once degraded, the DNA disappeared, leaving no detectable trace of degradation after 1 hr. It is noteworthy that after the smear was detected, macronuclear fragments did not disappear (Fig. 4). The smears detected in this study, therefore, indicate the occurrence of partial autolysis of macronuclear fragments.

Detection of DNA smears in single lanes in 3 cultures (Fig. 3A, Fig. 5A, B) is an indication that nuclear events proceeded highly synchronously through 40 hr of conjugation. If the nuclear events were to proceed less synchronously, smears would not appear in a single lane. Rather, smears would not be detected at all, which might be what occurred in most of the cultures examined. The short-tailed smears in successive lanes of 40, 41 and 42 hr cultures (Fig. 5C) may indicate incomplete synchronization.

Why DNA degradation occurred as late as 33, 38 and 40 hr after the chemical induction of conjugation is unknown. In both *P. caudatum* (Mikami, 1975) and *P. multimicronucleatum* (Takagi, Y., unpublished observations), MR is induced by transferring conjugating pairs or early exconjugants to culture medium; longer starvation of exconjugants prevents MR. The macronuclear fragments to be regenerated should remain intact, because if partial autolysis of the fragments resulted in their genomic incompleteness, they would not be able to regenerate a functional macronucleus. It is probable, therefore, that the partial autolysis detected in starved exconjugants around 40 hr after the chemical induction may indicate the critical point after which MR becomes impossible or can not

produce viable progeny. To verify the hypothesis, we have to establish beforehand an experimental system that can induce 100% occurrence of DNA degradation and MR. Also in *P. tetraurelia* in which we failed to detect DNA degradation, we are to assess DNA degradation in synchronized autogamous cells collected every hour.

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