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Unequal Gene Amplification and Transcription in the Macronucleus of *Tetrahymena pyriformis*†

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ABSTRACT: Deoxyribonucleic acid (DNA) repetition classes of the micro- and macronuclei of *Tetrahymena pyriformis* were analyzed by reassociation kinetics. The most slowly reassociating class of DNA, which accounted for ~80% of the total micronuclear DNA, had a $C_0t_{1/2}$ value of ~200. This value corresponds well to that for the single-copy (nonrepetitive) sequences with a genome size of 0.4 pg of DNA per diploid micronucleus. There were other sequences reannealing a hundred times or several hundred times faster, each constituting ~10% of the micronuclear DNA. They are assumed to represent moderately and highly repeated sequences in this genome. On the other hand, macronuclear DNA was composed of nearly only one repetitive class whose $C_0t_{1/2}$ value was 200, similar to the value for the single-copy class DNA of the micronucleus. This indicates that most, if not all, of the macronuclear DNA exists as rather uniformly repeated sequences with a frequency equal to the DNA ratio of macro-/micronucleus. This ratio has been reported to be 23 (Doerder et al., 1977; Williams et al., 1978). We conclude from these results that, when a macronucleus develops from the micronucleus, the single-copy sequences amplify by more than 20 times, while the repeated sequences remain virtually unamplified or amplify to a much lesser extent, a few times at most. Hybridization experiments with DNA complementary to poly(A)-containing ribonucleic acid (RNA) and micro- or macronuclear DNA indicate that, although all three classes of DNA in the micronuclear genome are transcribed into messenger ribonucleic acid (mRNA), the sequences transcribed from moderately repeated DNA accounting for only a few percent of the macronuclear DNA make up more than 30% of the mRNA, suggesting the higher production and/or stability of these sequences.

The ciliated protozoan *Tetrahymena pyriformis* possesses a transcriptionally inactive, diploid, germinal micronucleus and a transcriptionally active, polyploid, somatic macronucleus. In vegetatively growing cells, the micronucleus divides mitotically, whereas the macronucleus divides amitotically. During the course of sexual conjugation, the macronucleus is destroyed and then resorbed, while the micronucleus undergoes meiosis. After subsequent reciprocal exchange and fusion of the two haploid genetic nuclei, a new macronucleus develops through a process of amplification of micronuclear DNA to ~45-ploid (Woodard et al., 1972; Doerder et al., 1977). The multiple copies of rRNA genes in the macronucleus are generated by amplification of the apparently single gene in the micronucleus (Yao & Gall, 1977), reaching a higher multiplicity than other DNA sequences (Yao et al., 1974). On the contrary, renaturation kinetics of macro- and micronuclear DNA were reported to be very similar, suggesting that no significant unproportional gene amplification occurred on the extranucleolar genomic DNA except that some 10–20% of the micronuclear DNA sequences appeared to be absent in macronuclear DNA (Yao & Gorovsky, 1974).

We reexamined the reassociation kinetics of these DNAs with our highly purified micro- and macronuclear preparations and with the aid of highly labeled DNA markers prepared by the nick translation technique which had been developed by Rigby et al. (1977). The results obtained indicate that the

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sequence differences between micro- and macronuclear DNA are much larger than reported previously, indicating that a differential amplification mechanism exists for different repetitive DNA classes. We then studied the transcription of these different classes of DNA during the vegetative growth of this organism by hybridizing highly labeled DNA complementary to total poly(A+) RNA[1] with a large excess of micro- or macronuclear DNA. The data indicated that although the transcripts represent most of the different frequency classes of the micronuclear genome, repeated DNA classes were represented in a relatively large fraction of mRNA.

Materials and Methods

Culturing of Cells. *T. pyriformis* (variety I, mating type IV) was cultured in 2% proteose peptone, 1% yeast extract, and 0.6% glucose. Five liters of culture was maintained at room temperature for 2 or 3 days. Cells were harvested in the late exponential phase.

Isolation of Nuclei. Cultures containing (2–5) $\times 10^5$ cells/mL were harvested, and cells were lysed carefully by gentle stirring in a medium containing 0.4% Triton X-100, 10 mM $MgCl_2$, and 0.25 M sucrose. After the solution was adjusted to 2 M sucrose by addition of solid sucrose, nuclei were isolated by centrifugation at 21 000 rpm for 2 h at 2 °C. The nuclear pellet was resuspended in 0.25 M sucrose containing 10 mM $MgCl_2$ and passed through a Nucleopore filter (N-111-02) with a pore size of 5 μm (Gorovsky et al., 1975). The filtration was effected just by gravity. The macronuclei were retained completely on the filter while micronuclei passed through it. Macronuclei were collected by washing the filter with the same sucrose–Mg^{2+} medium, and micronuclei were obtained from the filtrate by centrifugation at 3000 rpm for 30 min. Contamination of micronuclei in the macronuclear preparation was negligible. Contamination of the micronuclear preparation by recognizable macronuclei was not detected under the light microscope. Under the electron microscope, however, though no intact macronuclei were detected, small amounts of chromatin bodies and fragments of nucleoli were detected in the preparation. It was found to be important to handle nuclei as gently as possible during the course of isolation in order to minimize the contamination of these particles in the micronuclear preparation. Especially, stirring of cells during lysis should not be vigorous. Otherwise, nucleoli located on the periphery of macronuclei tend to become detached and contaminate the micronuclear preparation.

Preparation and Shearing of DNA from Micro- and Macronuclei. Nuclear preparations were suspended in 10 volumes of SET buffer (0.15 M NaCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.4). Proteinase K and $NaDodSO_4$ were added to final concentrations of 200 μg/mL and 0.2%, respectively, and the mixture was incubated at 55 °C for 60 min. An equal volume of water-saturated phenol was then added, and the mixture was shaken for 30 min at room temperature. After centrifugation, the water phase was extracted with phenol 3 times and then precipitation was carried out with 2 volumes of ethanol. The precipitate was resuspended in SET buffer and digested with 50 μg/mL RNase A and 20 μg/mL RNase T_1 (both treated at 90 °C for 5 min) at 37 °C for 60 min. Then, $NaDodSO_4$ and proteinase K were added to final concentrations of 0.2% and 50 μg/mL, respectively, and incubation was continued at 55 °C for 60 min. After two phenol extractions, the aqueous phase was precipitated with ethanol. Ethanol precipitation was repeated 3 times, and the final precipitates were dissolved in doubly distilled water.

DNA was sheared into 300–500 base pairs by sonication with a Kubota KMS-250 sonicator at the maximum output for 5 min. The DNA chain length was determined by agarose gel electrophoresis with restriction endonuclease *Hin*dIII fragments of SV40 DNA as the markers. For preparation of labeled DNA by nick translation (see below), sonication was only for 2 min. After the labeling procedures, the sizes of the DNA fragments were found to be 400–600 base pairs by gel electrophoresis (data not shown).

Labeling of Micro- and Macronuclear DNAs. Micronuclear DNA was labeled with [^{3}H]dCTP and [^{3}H]dTTP and macronuclear DNA with [^{32}P]dCTP as precursors by the nick translation technique (Rigby et al., 1977). In some experiments, this labeling was reversed to exclude the effect of fragment sizes with different isotopes. ^{3}H-Labeled DNA had specific activities of the order of 5×10^6 cpm/μg of DNA, whereas those for ^{32}P-labeled DNA were of the order of 2×10^7 cpm/μg of DNA.

Labeling of Repetitive and Nonrepetitive Micronuclear DNA. Sheared and denatured micronuclear DNA was incubated to a C_0t of 1×10^2, and single- and double-stranded DNAs were separated on a column of hydroxylapatite. Single-stranded DNA was hybridized extensively (to a C_0t of 1×10^4) to obtain double-stranded DNA. These DNAs were labeled by the nick translation technique with either [^{3}H]dCTP or [^{32}P]dCTP as precursor.

Preparations of Poly(A+) RNA and Synthesis of cDNA. For preparation of the total poly(A)-containing messenger ribonucleic acid [poly(A+) mRNA], cells were homogenized in 15 volumes of NETS buffer [50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 10 mM EDTA, 10 μg/mL sodium heparin, and 0.5% $NaDodSO_4$] and immediately extracted with an equal volume of a phenol–chloroform (1:1) mixture containing 0.1% 8-hydroxyquinoline. Nucleic acid was precipitated with ethanol, dissolved in NETS buffer containing 0.4% $NaDodSO_4$, and digested with 100 μg/mL proteinase K at 37 °C for 45 min. After the phenol extraction and ethanol precipitation, the precipitate was dissolved in NETS buffer containing 0.57 g/mL CsCl, layered on a pad of CsCl (0.96 g/mL), and centrifuged at 35 000 rpm for 18 h (Glišin et al., 1974). The pelleted RNA was suspended in 50 mM Tris-HCl (pH 7.6), 0.7 M NaCl, 10 mM EDTA, and 28% formamide and passed through a column (0.5 × 3.5 cm) of poly(U)–Sepharose (Pharmacia Fine Chemicals) (Lindberg & Persson, 1972). The retained poly(A+) RNA was eluted with 90% formamide, 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, and 0.2% sodium dodecyl sarcosinate. This procedure was repeated twice.

The reaction mixture for the cDNA preparation contained, in a total volume of 0.05 mL, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 2 mM dithiothreitol, 100 μg/mL actinomycin D, 260 μg/mL poly(A+) RNA, 50 μg/mL oligo(dT)$_{10}$, [^{32}P]-dCTP (260 Ci/mmol, Radiochemical Centre), 0.2 mM of three other nonradioactive deoxynucleoside triphosphates, and ~20 units of avian myeloblastosis virus (AMV) reverse transcriptase (Sakai et al., 1978). After incubation at 37 °C for 90 min, cDNA was purified by phenol–chloroform extraction and alkaline treatment (0.3 N NaOH, 3 h, 60 °C), followed by passage through a column (0.5 × 10 cm) of Sephadex G-50. The specific radioactivity of the ^{32}P-labeled cDNA was $\sim 2 \times 10^8$ cpm/μg.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; poly(A+) RNA, poly(A)-containing RNA; cDNA, complementary DNA. Enzymes used were RNase T_1 (EC 2.7.7.26), RNase A (EC 2.7.7.16), and nuclease S_1, a single-strand-specific nuclease from *Aspergillus*.

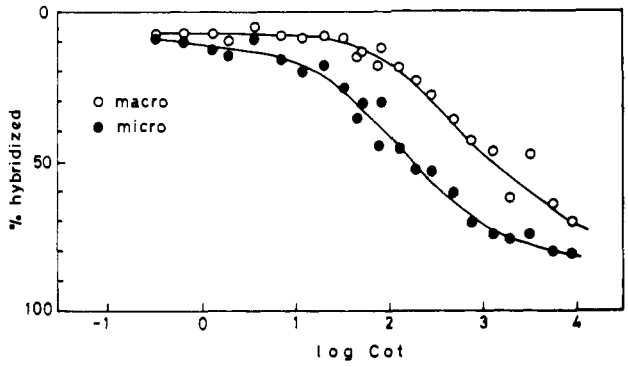

FIGURE 1: Hybridization of micro- and macronuclear DNA with a large excess of micronuclear DNA. 6000 cpm of ^{3}H-labeled micronuclear DNA (8 ng) and 3500 cpm of ^{32}P-labeled macronuclear DNA (1.6 ng) were mixed and incubated with either 23 or 0.49 μg of micronuclear DNA in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA at 65 °C up to the indicated C_0t values. Labeled micro- and macronuclear DNA served as internal standards for each other. After incubation, the hybridization mixture was diluted with 1.0 mL of S_1 buffer [30 mM sodium acetate (pH 4.5), 0.15 M NaCl, and 1 mM $ZnCl_2$] and divided into two parts. One part was digested with nuclease S_1, and the other was incubated in the same manner but without nuclease S_1. Trichloroacetic acid insoluble radioactivity was counted for both samples, as described under Materials and Methods, from which the percentage of the total radioactivity that was in the hybrid was calculated. (●) ^{3}H-Labeled micronuclear DNA; (○) ^{32}P-labeled macronuclear DNA.

Hybridization Procedures. DNA–DNA hybridization was performed in 5–10 μL of 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA containing ~3000–6000 cpm of nick-translated micro- or macronuclear DNA and an appropriate amount of micro- or macronuclear DNA (Sakai et al., 1978). After denaturation at 100 °C for 10 min, the mixture was incubated at 65 °C for an appropriate period. After incubation, the hybridization mixture was diluted with 1 mL of S_1 buffer [30 mM sodium acetate (pH 4.5), 0.15 M NaCl, and 1 mM $ZnCl_2$] and incubated with 400 units of nuclease S_1 (Seikagaku Kogyo, Japan) in the presence of 10 μg of denatured rainbow trout DNA at 37 °C for 90 min. Nuclease-resistant radioactivity was precipitated with 10% trichloroacetic acid and counted on a glass filter disk. In the case of cDNA vs. micro- or macronuclear DNA hybridization, ~20 000 cpm of cDNA was mixed with a large excess of sheared, alkali-denatured micro- or macronuclear DNA. Their amounts are given in the figure legends. Sealed samples were denatured again in boiling water for 10 min and incubated at 65 °C up to their appropriate C_0t values. The mixtures were then treated with 400 units of nuclease S_1 and precipitated with 10% trichloroacetic acid. Hybridized cDNA was collected on a glass filter disk and the ^{32}P radioactivity counted.

Results

Micronuclear DNA Driven Reaction. To study the repetitive DNA classes of the *Tetrahymena* micro- and macronucleus, we first analyzed micronuclear DNA driven reactions by using labeled micro- and macronuclear DNA as monitoring components. Labeled DNAs were mixed with a large excess of unlabeled micronuclear DNA and incubated to the intended C_0t values (Britten & Kohne, 1968), and the percentages of duplexes formed were determined by nuclease S_1 digestion. As shown in Figure 1, renaturation kinetics for micronuclear DNA indicate that there are different repetitive classes of DNA in the micronucleus. The major one accounting for some 80% of the micronuclear DNA had a $C_0t_{1/2}$ of ~200. This value is just as expected for nonrepetitive DNA with the genome size of the micronucleus, 0.2 pg/haploid (Straus, 1976; Williams et al., 1978). This means that ~80% of the genome is composed of single-copy sequences, although whether they exist literally once or a few times cannot be conclusively determined with the $C_0t_{1/2}$ value alone. The remaining 20% of the DNA reannealed much faster: half of which did so about hundred times faster and the other half several hundred to a thousand times faster than the major single-copy sequences. These rapidly reassociating fractions are thought to represent repeated DNA classes with different degrees of repetition.

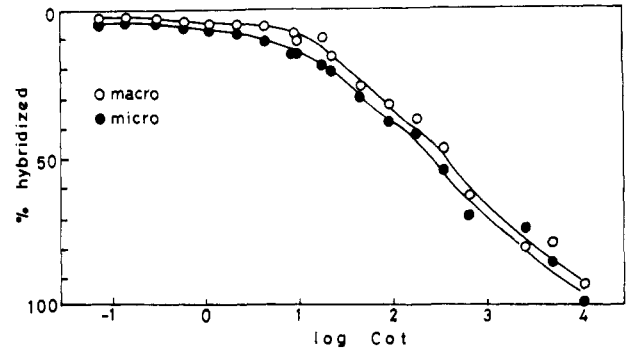

FIGURE 2: Hybridization of micro- and macronuclear DNA with a large excess of macronuclear DNA. 6000 cpm of ^{3}H-labeled micronuclear DNA (8 ng) and 3500 cpm of ^{32}P-labeled macronuclear DNA (1.6 ng) were mixed and incubated with either 53.6 or 0.113 μg of macronuclear DNA in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA at 65 °C up to the indicated C_0t values. Subsequent procedures were the same as those described in Figure 1. (●) ^{3}H-Labeled micronuclear DNA; (○) ^{32}P-labeled macronuclear DNA.

When the reassociation kinetics were monitored with labeled macronuclear DNA, they showed a rather monophasic curve with a $C_0t_{1/2}$ value of ~200, similar to that of single-copy sequences in the micronucleus. This indicates that the repeated sequences in micronuclear DNA are scarcely represented in macronuclear DNA and that macronuclear DNA consists largely of single-copy sequences of micronuclei which have amplified ~23 times rather uniformly. Here we assume the DNA ratio in the micro- and macronucleus to be 1:23 (Doerder et al., 1977; Williams et al., 1978).

Macronuclear DNA Driven Reaction. To analyze further both DNA components, we studied the reassociation kinetics driven by macronuclear DNA (Figure 2). In contrast to the micronuclear driven reaction, the C_0t curves were nearly monophasic whether micro- or macronuclear DNA was used as the labeled component. Most of the macronuclear DNA was composed of one complexity class whose $C_0t_{1/2}$ was ~200, although the curve was somewhat broader than the theoretical single-component one. Less than 5% of the macronuclear DNA appeared to reanneal at C_0t values of highly and moderately repeated DNA classes. When labeled micronuclear DNA was used as the probe, a slightly higher percentage of labeled micronuclear DNA than labeled macronuclear DNA formed duplexes at a low macronuclear C_0t (10–20). We interpret this as reflecting the presence of repeated sequences in micronuclear DNA at a higher percentage than in macronuclear DNA. Some self-reassociation of labeled micronuclear DNA may also have taken place. When the labeling was reversed, i.e., micronuclear DNA was labeled with ^{32}P and macronuclear DNA with ^{3}H, this small difference was again observed, indicating that it was not due to the different fragment sizes caused by different isotopes. In view of the almost complete duplex formation of the labeled micronuclear DNA with an excess of macronuclear DNA, the elimination of some sequences in micronuclear DNA during development of macronuclei appears to be relatively small and was estimated to be less than 10%.

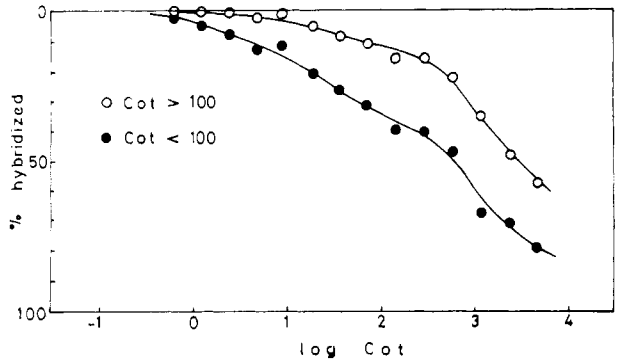

FIGURE 3: Hybridization of repetitive and single-copy micronuclear DNA with a large excess of micronuclear DNA. Repeated (C_0t 100) and single-copy (C_0t 100) micronuclear DNAs were separated on a hydroxylapatite column and labeled by nick translation as described under Materials and Methods. 3000 cpm of ^{32}P-labeled repetitive micronuclear DNA (5 ng) and 4000 cpm of ^{3}H-labeled single-copy micronuclear DNA (14 ng) were mixed and incubated with 1.8–72 μg of micronuclear DNA in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA at 65 °C up to the indicated C_0t values. Subsequent procedures were the same as those in Figure 1. (●) ^{32}P-Labeled repeated micronuclear DNA; (○) ^{3}H-labeled single-copy micronuclear DNA.

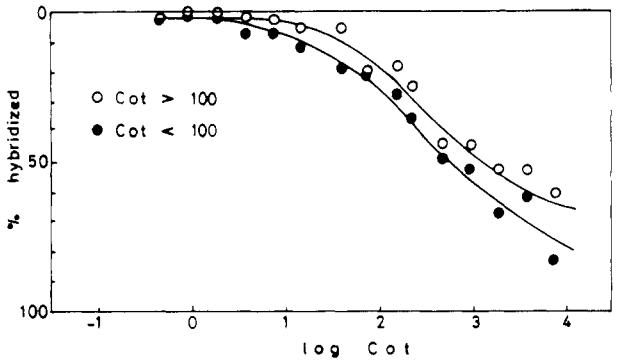

FIGURE 4: Hybridization of repeated and single-copy micronuclear DNA with a large excess of macronuclear DNA. The procedures were the same as those in Figure 3, except that 0.113–11.3 μg of macronuclear DNA was used as the driver. (●) ^{32}P-Labeled repeated micronuclear DNA; (○) ^{3}H-labeled single-copy micronuclear DNA.

Reactions with Fractionated DNA. To confirm the presence of different repeated classes of DNA in the *Tetrahymena* genome, we fractionated the labeled micronuclear DNA on a hydroxylapatite column into repeated and nonrepeated (single copy) fractions: the former annealing with a C_0t of 100 and the latter remaining single-stranded at the same C_0t. Each fraction was treated with a large excess of either micronuclear or macronuclear DNA (Figures 3 and 4). In the reaction driven by micronuclear DNA, the repeated class of micronuclear DNA behaved completely differently from the nonrepeated class, showing a much higher reassociation rate (Figure 3). It appears that this fraction also consists of more than one (highly to moderately) repeated DNA class. A portion of this fraction behaved as the nonrepeated class, probably due to the contamination by the single-copy class DNA. On the other hand, the nonrepeated fraction thus isolated behaved mostly as single-copy DNA, although a small portion of this fraction annealed faster, probably because of the contaminating repeated DNA classes. In contrast, the reaction kinetics, when driven by macronuclear DNA, showed very similar monophasic patterns with both repeated and nonrepeated DNA, although the former had a slightly depressed shoulder (Figure 4). These results may be explained by the assumption that macronuclear DNA contained only very small amounts of repeated DNA classes of the micronuclear genome. Much of the reaction with labeled repeated DNA in Figure 4 may represent that of contaminating nonrepeated DNA. In any case, these results corroborate the previous results obtained with unfractionated DNA (Figures 1 and 2) and suggest an unequal amplification mechanism for different DNA classes in micronuclear DNA during the development of the macronucleus. That is, the single-copy DNA is amplified to a greater extent, while the repeated DNA classes remain unamplified or are amplified to a much lesser extent. Thus, the macronuclear DNA is composed mostly (~95%) of sequences with a similar repetition number, as shown by its rather monophasic reassociation kinetics.

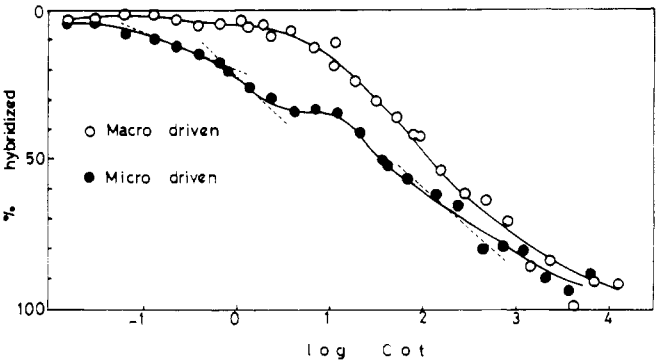

FIGURE 5: Hybridization of cDNA complementary to cellular poly(A)-containing mRNA with a large excess of micro- or macronuclear DNA. 20 000 cpm of ^{32}P-labeled cDNA complementary to cellular poly(A+) mRNA (0.056 ng) was mixed with either 0.048–14.6 μg of micronuclear DNA or 0.048–56 μg of macronuclear DNA and incubated in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA at 65 °C up to the indicated C_0t values. After incubation, the hybridization mixture was divided into two parts. One part was digested with nuclease S_1, and the other was incubated in the same manner but without nuclease S_1. Trichloroacetic acid insoluble radioactivity was counted for both samples as described under Materials and Methods, from which the percentage of the total radioactivity that was in the hybrid was calculated. (●) Micronuclear DNA driven reaction; (○) macronuclear DNA driven reaction.

Transcription of Different Classes of DNA. The gene expression in *T. pyriformis* is mediated almost exclusively by the polyploid somatic macronucleus. To gain some insight into the transcriptional significance of the unproportional amplification in macronuclear DNA described above, we tried to determine what repeated classes of DNA were actually transcribed as mRNA in the cytoplasm. For this purpose, we extracted total RNA from the cell and purified poly(A+) RNA by means of poly(U)–Sepharose column chromatography. Complementary DNA (cDNA) was synthesized by reverse transcription using this total poly(A+) RNA as the template. The size of the synthesized cDNA, which was determined by sucrose density gradient centrifugation, was ~5–7 S (data not shown). Hybridization was carried out between the cDNA and a large excess of micro- and macronuclear DNA (Figure 5). The hybridization profile of cDNA and a large excess of micronuclear DNA had apparently three transitions. About 15% of the cDNA was complementary to the highly repeated class of the micronuclear DNA whose $C_0t_{1/2}$ value was ~0.4, 20% of the cDNA was complementary to moderately repeated sequences of the micronuclear DNA whose $C_0t_{1/2}$ value was ~2, and the remaining 60% or more of the cDNA was complementary to the nonrepeated sequences of the micronuclear DNA whose $C_0t_{1/2}$ value was ~200. On the contrary, the hybridization profile of cDNA driven by macronuclear DNA showed almost one complexity class with a $C_0t_{1/2}$ value of ~200, except that only a few percent appeared to represent the moderately repeated class, resembling

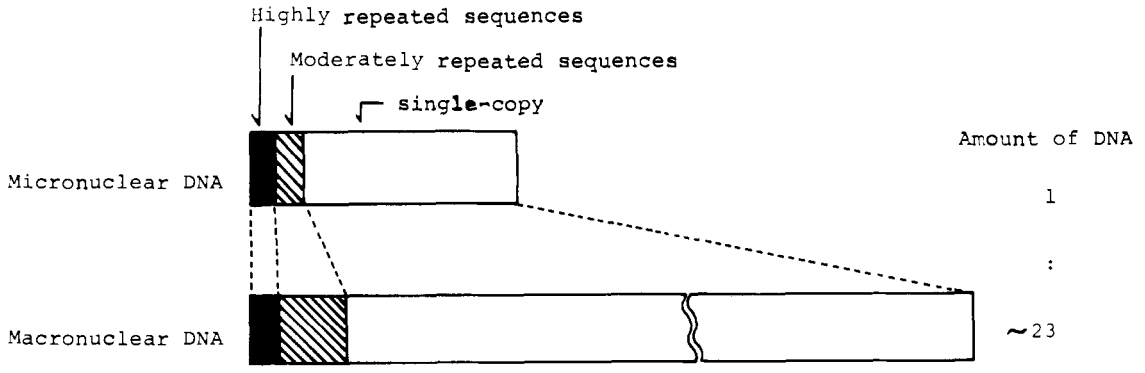

FIGURE 6: Schematic representation of gene amplification in *Tetrahymena*. Highly and moderately repeated sequences in the micronucleus constituting 20% of the genome replicate little or only a few times during the development of a macronucleus in which single-copy sequences amplify ~23 times and make up more than 95% of the macronuclear DNA.

the reassociation kinetics for macronuclear DNA. These results indicate that poly(A+) RNA of *Tetrahymena* is transcribed rather uniformly from all the repeated sequences of macronuclear DNA, most of which are amplified from the single-copy class of micronuclear DNA. The reassociation kinetics, when driven by micronuclear DNA, also indicate that the sequences transcribed from nonamplified DNA derived from the highly to moderately repetitive micronuclear geneomic DNA are relatively abundant (~35%) in poly(A+) RNA, albeit the sequences represent only a very small fraction of the macronuclear DNA.

Discussion

In this study, we first compared the DNA of micro- and macronuclei of *T. pyriformis* by analysis of the reassociation kinetics. The results indicate that, contrary to the previous report (Yao et al., 1974), the composition of the macronuclear DNA is remarkably different from that of the micronuclear DNA.

Whereas the micronuclear DNA consists of significant amounts of highly repeated, moderately repeated, and single-copy sequences (Figures 1 and 4), the macronuclear DNA is composed mainly of one moderately repeated class of DNA whose $C_0t_{1/2}$ value is close to that of the single-copy sequences of the micronucleus, although the presence of a few percent of highly repeated sequences is also apparent (Figure 2). The $C_0t_{1/2}$ value of the single-copy sequences, 200, is quite reasonable for the calculated genome size (0.2 pg/haploid) of this species (Williams et al., 1978).

Although Yao & Gorovsky (1974) concluded from their analysis of reassociation kinetics that 80–90% of the sequences present in micronuclei were present in similar amounts in macronuclei, the repetitive classes of these sequences and the differences were not referred to, probably because of the lack of a clear-cut difference in the C_0t curves between micro- and macronuclear DNA. If carefully examined, however, their curve of the micronuclear-driven reaction shows a somewhat similar pattern to ours, shown in Figure 1, though the difference between the two curves monitored with labeled micro- and macronuclear DNA is not so marked as in our study. This discrepancy may be due to the lower ratio (10:1) of micro-/macronuclear DNA employed in their micronuclear-driven reactions. A small amount of contaminating macronuclear DNA in the micronuclear DNA may also cause this effect. Also, the possibility of strain difference cannot be ruled out either.

In any case, our present study has disclosed the marked differences between micro- and macronuclear DNA of *Tetrahymena* for the first time by using a real *vast excess* of driving DNA which is now feasible with the use of nick-translated highly labeled DNA. Furthermore, these differences were confirmed by fractionating micronuclear DNA into repeated and nonrepeated sequences and retreating them with micro- and macronuclear DNA.

The following scheme of gene amplification in *Tetrahymena* emerges from the above-mentioned results (Figure 6). During the macronuclear development from the micronucleus after conjugation, single-copy sequences constituting ~80% of the micronuclear DNA replicate many times to make up more than 90% of the macronuclear DNA, the amount of which is supposed to be 23 times that of the micronucleus (Doerder et al., 1977; Williams et al., 1978). On the other hand, highly and moderately repeated sequences accounting for 20% of micronuclear DNA either do not replicate or replicate only a few times on average to constitute a small fraction (several percent) of macronuclear DNA.

Some of the highly repeated DNA in the micronucleus might be a satellite-type DNA which is concerned with mitotic movement of chromosomes of micronuclei. Such a DNA may not be necessary so much in the macronucleus which divides amitotically and could even be discarded partially when a macronucleus develops. Other repeated classes may represent some regulatory sequences for structural genes. These sequences may not have to be amplified to the same extent as the unique structural genes themselves. The differential amplification demonstrated in this study suggests the occurrence of reorganization of repeated and single-copy DNA sequences in the macronucleus before effective transcription and expression of the genome occur. This could probably be demonstrated by the patterns of restriction fragments of DNA that are hybridizable with some single-copy or repeated genes. Such a study is now under way.

Next, we analyzed the expression of these different classes of DNA in mRNA. Cellular poly(A+) RNA, tentatively assumed to be mRNA, was found to be transcribed from macronuclear DNA derived from nearly all three categories of micronuclear DNA: i.e., highly, moderately, and nonrepeated sequences. It is interesting to note that, nevertheless, highly or moderately repeated sequences make up only less than 10% of the macronuclear DNA and the transcripts from these sequences constitute more than 30% of poly(A+) RNA. These results suggest either that these repeated sequences are transcribed more actively than single-copy sequences are as a whole, or that the poly(A+) RNA from repeated sequences is more stable than that transcribed from single-copy sequences or both. Further study is necessary to clarify these points.

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Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonuclease†

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ABSTRACT: Intact ribonucleic acid (RNA) has been prepared from tissues rich in ribonuclease such as the rat pancreas by efficient homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol to break protein disulfide bonds. The RNA was isolated free of protein by ethanol precipitation or by sedimentation through cesium chloride. Rat pancreas RNA obtained by these means has been used as a source for the purification of α-amylase messenger ribonucleic acid.

The preparation of undegraded ribonucleic acid from a number of cell types is hindered by the presence of active nucleases. An extreme example of this is the rat pancreas which contains over 200 μg of ribonuclease A per g of tissue wet weight (Beintema et al., 1973). Within the pancreatic exocrine cells, ribonuclease A as well as other digestive enzymes and zymogens appears to be synthesized on ribosomes bound to the cytoplasmic face of the endoplasmic reticulum, extruded directly into the cisternal side, and subsequently packaged in secretory granules. Thus, the functions of the cytosol are effectively sequestered from these strong hydrolytic activities. Disruption of the cells, however, inevitably results in rapid mixing of RNA and RNase.[1,2] One way to eliminate nucleolytic degradation of RNA is to denature all of the cellular proteins including RNase. This approach would be successful only if the rate of denaturation exceeds the rate of RNA hydrolysis by RNase. Deproteinization procedures using guanidine hydrochloride (Cox, 1968) or phenol even in the presence of RNase inhibitors such as heparin, iodoacetate, and detergent (Parish, 1972) are insufficiently effective to yield intact RNA from the pancreas.

We describe here a generally applicable method for the quantitative isolation of intact RNA. The rate of denaturation is maximized by the combined use of a strong denaturant, guanidinium thiocyanate, in which both cation and anion are potent chaotropic agents (Jencks, 1969), and a reductant to break protein disulfide bonds which are essential for RNase activity (Sela et al., 1956). This method has been employed in the isolation of intact biologically functional RNA from rat pancreas and the purification of mRNA for α-amylase, the most abundant pancreas-specific protein (Sanders & Rutter, 1972).

Experimental Procedure

Chemicals and Solutions. All glassware was rendered nuclease free by overnight treatment at 180 °C. Whenever possible [see Ehrenberg et al. (1974)], stock solutions were treated for 20 min with 0.2% diethyl pyrocarbonate and then thoroughly boiled to remove traces of the reagent. Buffers such as tris(hydroxymethyl)aminomethane, which contains a primary amine that reacts with diethyl pyrocarbonate, were avoided.

Guanidinium thiocyanate stock (4 M) was prepared by mixing 50 g of Fluka purum grade guanidinium thiocyanate (Tridom, Inc., Hauppage, NY) with 0.5 g of sodium *N*-lauroylsarcosine (final concentration 0.5%), 2.5 mL of 1 M sodium citrate, pH 7.0 (25 mM), 0.7 mL of 2-mercaptoethanol (0.1 M), and 0.33 mL of Sigma 30% Antifoam A (0.1%).

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[1] A brief note describing a version of this method has been published (Ullrich et al., 1977).
[2] Abbreviations used: RNase, ribonuclease; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid.

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