SELECTIVE AMPLIFICATION OF REPETITIVE DNA IN THE EUKARYOTE, TETRAHYMENA

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SUMMARY: Selective amplification of the repeated DNA sequences (ERRF) of the genome of the ciliated protozoan, Tetrahymena pyriformis W can be induced by periodic heat shock treatment of populations of cells in the circadian-infradian mode of growth. The amplified DNA sequences are replicated from pre-existing repetitive DNA sites within the genome as revealed by DNA/DNA kinetic-hybridization experiments.

INTRODUCTION:

Gene amplification is a recently discovered phenomenon which is apparently restricted to the cells of higher organisms (Brown and Dawid, 1968). There is considerable evidence that amplification occurs at DNA sites within the eukaryotic DNA complement which are genetically redundant (Gall, 1969; Ritossa and Scala, 1969) as are the ribosomal RNA cistrons (Miller, 1966). The relation between unstable gene redundancy and dosage compensation and the physiological control of gene amplification has been examined in the dipteran metazoan, Drosophila (Tartoff, 1970). These intriguing studies suggest that the level of ribosomal genes is physiologically determined during embryonic development.

In the present study, some of the factors which influence the replication of the repeated DNA sequences of the genome of the single-celled, eukaryotic protozoan, Tetrahymena pyriformis W were examined. They are the cell generation time of batch culture populations, and periodic heat shock treatment.

MATERIALS AND METHODS:

Culture of T. pyriformis: Batch cultures of the ciliated protozoan, Tetrahymena pyriformis, the amicronucleate strain, W, were grown axenically on either enriched proteose peptone medium (Szyszko, et al, 1968) or on defined medium supplemented with 0.4% proteose peptone (Brunk, 1967). Chemicals and reagents: 5-^{3}H-thymidine (6.7 Ci/mM) was purchased from New England Nuclear Corp. (Boston, NEN); S&S filters (type B6, 25 mm) from Brinkman Instruments, Inc., Sartorius Division; Agarose biogel (A-150m, 50-100 mesh), acrylamide gel (P-100), hydroxyl-apatite (Biogel HTP), and ribonuclease, salt-free, all from Calbiochem Corp., Los Angeles, Cal. SSC is 0.15 M NaCl + 0.015 M Na-citrate (pH=7); 2X and 6X are 2 and 6 times as concentrated as SSC. FOSC is 33% formamide (V/V) in 2X SSC. PM is preincubation medium and contains 0.1% bovine serum albumin in FOSC. Labeling of synchronous and asynchronous cultures: Induction of synchronous cell division of cultures in the ultradian mode of growth (Ehret and Wille, 1970) was accomplished by a slight modification of the cyclic heat shock regime previously developed for mass cultures of Tetrahymena (Scherbaum and Zeuthen, 1954). For labeling experiments, cells were grown either in EPP or defined media to a titer of 50-100 x 10^{4} cells/ml and the radioisotope added at different times during the synchronizing treatment (see figure 1, hs-u, and hs-i), or radioisotope was added to batch cultures either in rapid exponential phase of growth (ultradian mode) or in the slow growth mode (circadian-infradian mode) for one or more cell generations (see figure 1, A,B, and C).

Separation of repeated (ERRF) and non-repeated (SRF) DNA: Tetrahymena DNA was purified by agarose gel filtration technique (Barnett, Wille, and Ehret, 1971), and sheared to uniform sized fragment of about 750,000 daltons in a Ribi cell fractionator (Sorvall Inc., Norwalk, Conn.) at 50 k psi pressure at 10°C, and concentrated by addition of dry biogel P-100 followed by vacuum filtration. Concentrated DNA solutions were heat

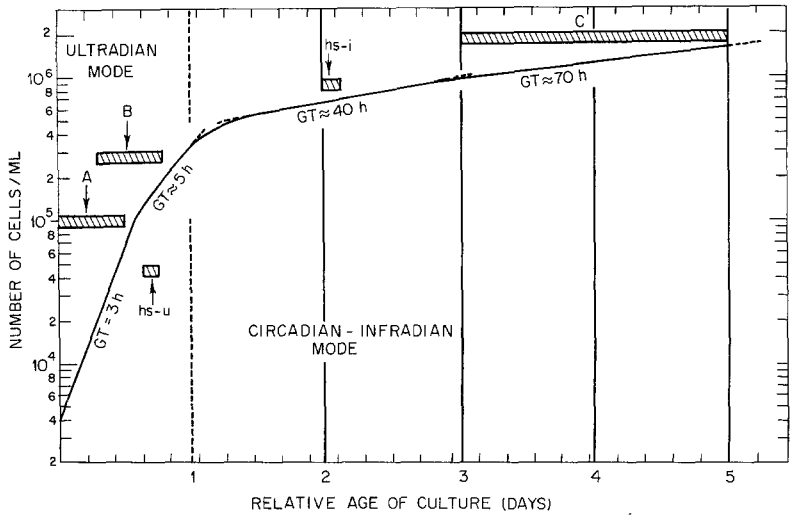
denatured (100° C, for 5 minutes in 0.2 X SSC), adjusted to 0.12 M in phosphate buffer and allowed to reassociate to a Cot (defined as product of initial concentration of DNA and time, expressed in Mol x sec./liter) of 100, at 44°C. The partially reassociated DNA was chromatographed on hydroxylapatite columns to separate the single-stranded DNA (SRF fraction, Britten and Kohne, 1966) from the double-stranded DNA (ERRF). SRF and ERRF DNA fractions were stored at -10°C for further use.

Attachment of DNA to filters: ERRF and SRF DNA solutions were alkali denatured and attached to filters as previously described (Barnett, Wille, and Ehret, 1971). Retention of ERRF DNA on filters was found (by using ^{3}H-thymidine-labeled ERRF DNA) to be 40%, while retention of SRF DNA was only 20%. A stack of 5 dried ERRF or SRF DNA filters were carefully cut into quarters, and two different groups of 5 "quartered filters" made 10 quartered filters used routinely for each experimental point.

DNA/DNA annealing conditions: All hybridizations involved various concentrations of ERRF and SRF DNA in FOSC annealed to DNA filters. To eliminate non-specific attachment of single-stranded labeled DNA, both blank filters and DNA filters were soaked in PM medium for 10 hours prior to exposure to radioactive DNA solutions (Denhardt, 1966).

RESULTS:

Replication of ERRF and SRF DNA in various stages of batch culture growth: Replication of the repeated and non-repeated DNA sequences of the Tetrahymena genome during the exponential phase of batch culture growth, and in the slow growth phase was followed by pulse-labeling cells according to the protocol given in figure 1. When DNA was prepared from cultures A,B, and C and the specific activities of ERRF and SRF DNA fraction assessed by spotting a known quantity of DNA on paper discs and counting in a liquid scintillation spectrometer, the results given in Table I were obtained. The rate of DNA synthesis is



1: A typical batch culture growth curve for Tetrahymena pyriformis W. Ordinate: Number of cells/ml; abscissa: relative age of culture. The figure shows five different experimental protocols for ^{3}H-thymidine labeling of cells in different stages of population growth. Protocols A and B, for labeling in the ultradian mode, and protocol C is for labeling in the circadian-infradian mode. Protocols hs-u and hs-i are for labeling during heat shock treatment in the ultradian, and circadian-infradian modes, respectively. The hatched area indicates the relative length of the labeling period in each case. The average generation time (GT) for the population at different stages of batch culture growth is given where slope change occur.

much greater in the ultradian mode of growth (cultured A, and B, figure 1), with approximately equal rates of synthesis for ERRF and SRF DNA sequences in both the ultradian and circadian-infradian modes of growth. Thus, it would appear that no differential rate of synthesis for the repeated versus the non-repeated DNA sequences occurs in either mode, although such might occur in the transitions between modes, as the proportion of ERRF DNA sequences to total genomic DNA is less in the circadian-infradian mode than in the ultradian mode of growth (Wille, unpubl.).

Effect of periodic heat shocks on ERRF and SRF replication: Two different protocols were followed to obtain DNA labeling during heat shock treatments, hs-u and hs-i as shown in figure 1. In protocol hs-u cells after reaching a titer of 5 x 10^4 cells/ml were subjected to a series of 30 minute temperature elevations (35°C) alternating with 30

TABLE I

Incorporation of ^{3}H-thymidine into the repeated and non-repeated DNA sequences of Tetrahymena under different growth conditions.

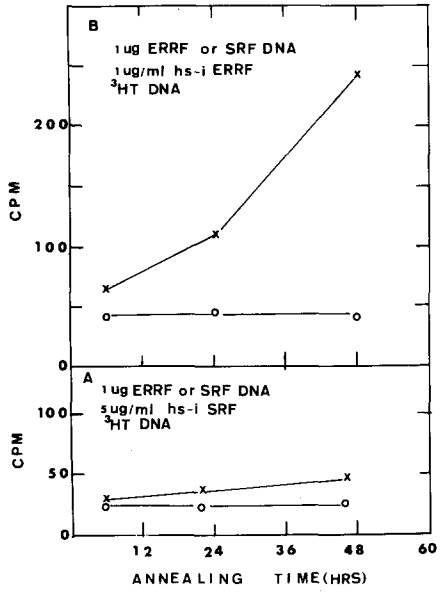
Stage of culture growth:	Specific Activities		ERRF/SRF
	ERRF(cpm/μg)	SRF(cpm/μg)	
Ultradian mode, A	1,428	1,128	1.3
Late ultradian mode, B	1,311	1,676	0.8
Circadian-infradian mode, C	433	487	0.9
Heat shock treatment:			
ultradian mode, hs-u	133	146	0.9
infradian mode, hs-i	2,576	139	18.5

minute periods at optimal growth temperature (27°C), and 5- ^{3}H-thymidine (5 μc/ml) added from the end of the third heat shock cycle to the end of the seventh heat shock cycle, at which time cells were harvested by centrifugation and ERRF and SRF DNA fractions prepared.

In protocol hs-i, cells were grown in defined medium and subjected to cyclic heat shock treatment as above, except ^{3}H-thymidine (5 μc/ml) was added between the third and sixth heat shock cycle. Further, it was noted that whereas a synchronous and approximate doubling of cell number was observed between 80-90 minutes after the last elevated temperature treatment in the hs-u protocol, this does not happen in the hs-i protocol. Labeled DNA was prepared from the cultures labeled as in protocol hs-i, and the specific activities of the ERRF and SRF DNA fractions obtained. Table 1 shows that while no preferential synthesis occurs during heat synchronizing treatments in the hs-u protocol, ERRF DNA is preferentially replicated (about 20-fold higher rate of synthesis) in the hs-i protocol.

Kinetics of hybridization of amplified DNA using ERRF and SRF DNA as template: Kinetic-hybridization curves for amplified DNA (hs-i ERRF and

its companion SRF fraction (hs-i SRF) on ERRF and SRF DNA filters is shown in figure 2. Amplified DNA hybridizes kinetically rapid on ERRF DNA (fig. 2B), and not at all on SRF DNA even after 40 hours annealing. After 11 days annealing nearly 50% of the input count from amplified DNA were bound on ERRF DNA filters, while less than 2% were bound to SRF DNA filters. Labeled SRF DNA (hs-i SRF) hybridized only barely on ERRF DNA filters (fig. 2A) and not at all on SRF DNA filters.



2: Kinetics of hybridization of amplified DNA annealed to either ERRF or SRF DNA filters. A, ^{3}H-thymidine-labeled hs-i SRF DNA (5 µg/ml) was annealed in 2.0 ml of FOSC with 1.0 ug of SRF per filter (o - o), or with 1.0 µg of ERRF per filter (x - x); B, ^{3}H-thymidine-labeled hs-i ERRF DNA (1 µg/ml) was annealed in 2.0 ml of FOSC with 1.0 µg of SRF per filter (o - o), or with 1.0 µg of ERRF per filter (x - x). Each point represents the mean of 5 filters. Blank filters (3/ml) gave background counts of 40 cpm, and 20 cpm in figs. 2B, and 2A, respectively, and superimpose on the SRF DNA filter counts in each case.

DISCUSSION:

This report describes the conditions under which gene amplification can be experimentally induced in the ciliate, Tetrahymena pyriformis. Two essential conditions must be fulfilled for this to occur, 1) cells be in the circadian-infradian mode of growth, and 2) application of cyclic heat shocks. Either condition by itself is not sufficient for

induction. The discussion of these findings pertains to the possible mode of action by which these two conditions act in concert to selectively permit replication of only the repetitive DNA sequences of the genome, and to the identification of the genes amplified under these conditions.

Several recent investigations (Elliot, Kennedy, and Bak, 1962; Cameron, Padilla, and Miller, 1966; and Nilsson and Leick, 1970) have shown that the cytological aspects of the macronucleus in Tetrahymena undergo changes when cells are either starved or subjected to cyclic heat treatment. Normally, log phase cells contain a macronucleus which has a large number of peripherally located nucleoli (approximately 750, Flickinger, 1965); these undergo fusion to form a fusion body following a 1 hour starvation period, and then disaggregate if the culture is refed (Nilsson and Leick, 1970). Fusion body formation also occurs when slowly dividing cultures are heat shocked as in the present study (Elliot, et al, 1962, and Cameron, et al, 1966). This reversible aggregation of nucleoli has been correlated with the cells capacity to synthesize new ribosomes, and ribosomal RNA synthesis (Cameron and Guile, 1965; and Nilsson and Leick, 1970). It has also been recently demonstrated that the nucleoli contain their own DNA which replicates in the G2 period of the cell cycle (Charret, 1969). We may surmise that these findings taken together suggest that the disaggregation of fusion bodies is accompanied by nucleolar DNA synthesis, which is the analogous situation reported for nucleolar DNA amplification in the african clawed-toad Xenopus laevis (Brown and Dawid, 1968). If so, our results would suggest that starved cells when heat shocked are predominately replicating nucleolar DNA. This is compatible with the fact that essentially all the ribosomal RNA cistrons of the Tetrahymena genome are located in the ERRF DNA sequences (Wille, Barnett, and Ehret, 1971), and the fact that heat shock treatment delays division by differential extension of the G2 period (Zeuthen, 1970). This expectation is

currently being tested by labeling experiments, to see if nucleolar DNA is preferentially replicated in starved cells that are refed, and by DNA/RNA hybridization experiments using "starved" and "refed" cells as source for ribosomal DNA sites complementary to ribosomal RNA. The latter experiments should detect different levels of rDNA sites in ERRF, if amplification occurs during refeeding.

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