PARTICIPATION OF RARE DNA TEMPLATES IN DNA/RNA HYBRIDIZATION IN THE EUKARYOTE, *TETRAHYMENA PYRIFORMIS**

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SUMMARY: The *in vivo* transcriptional capacities of the highly repeated (ERRF) and the low or nonrepeated (SRF) DNA nucleotide sequences of the ciliated protozoan, *Tetrahymena pyriformis* were examined by DNA/RNA hybridization. Both classes of DNA do serve as templates for RNA synthesis with fast reaction kinetics for ERRF and slow for SRF. Long-lived-ribosomal-enriched RNA retains the "fast" hybridizing property when hybridized to ERRF DNA and is hardly detectible on SRF DNA. Pulse-labeled RNA hybridizes well on both ERRF and SRF templates, but retains its "slow" hybridizing component only on SRF DNA and its "fast" hybridizing component only on ERRF DNA. The hybridization seen to date with *in vivo* pulse-labeled RNA reflects largely the reaction between ERRF templates and non-ribosomal RNAs, but both classes can be recognized in whole DNA versus whole RNA hybridizations.

INTRODUCTION: Recently it has become evident that the traditional methods of molecular hybridizations were mainly assessing the contribution of the redundant gene families in the DNA/DNA hybridization reaction, and the generic cross reactions of RNA transcripts with the redundant gene families in the DNA/RNA hybridization reaction.[1-6] There is some question as to whether the low or non-reiterated DNA sequences of the eukaryotic genome, in operational terms, the slowly reassociating fraction, SRF (see glossary, ref. 1) can even be detected by current hybridization techniques.[6-8] Although there have been recent efforts to detect and quantitate their participation in molecular hybridizations,[9-12] very little is known about the transcriptional capacity of rare genes in relation to cell development and differentiation. The associated question of the transcriptional capacity of the reiterated DNA sequences, in operational terms, the extremely rapidly reassociating fraction, ERRF,[1] is of interest since cells are clearly transcribing RNA from these variously repeated templates. It is of especial interest since the ERRF fraction of Tetrahymena

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DNA increases or decreases in proportion to the total DNA as a function of growth conditions.[13]

We have shown a "fast" and a "slow" hybridizing component in whole pulse-labeled RNAs in Tetrahymena versus unfractionated DNAs.[7] In the present study we report results on the hybridization of "pulse-labeled" RNA versus ERRF and SRF components from the DNA of slowly dividing cultures of *Tetrahymena pyriformis*.

MATERIALS AND METHODS:

Culture of *T. pyriformis*: Axenic cultures of the ciliated protozoan *Tetrahymena pyriformis* W, an amicronucleate strain, were grown in shaken batch cultues at 27°C and harvested while either still dividing several times a day (ultradian mode) or less than once a day (circadian-infradian mode)[14] as previously described.[7]

Chemicals: 5-^{3}H-Uridine (20 Ci/mM) was obtained from New England Nuclear Corp. (NEN, Boston) and from the International Chemical and Nuclear Corp. (ICN, Irvine); deoxyribonuclease, ribonuclease-free from Worthington Biochemical Corp., Freehold, N.J.; bovine pancreatic ribonuclease (salt-free); agarose biogel (150 million M.W., exclusion limit, 50-100 mesh) and polyacrylamide gel (P-100, 100,000 M.W., exclusion limit) all from Calbiochem. Corp., Los Angeles.

Labeled RNAs: Labeled stocks are designated primarily by the length of the pulse and secondarily by the cultural mode at the time of the pulse. Two different labeled stocks were employed in this study:

One hour pulse: 5 mCi of 5-^{3}H-uridine (21.7 Ci/mM, ICN) in 0.4 ml of ethanol was diluted with 20 ml of water and added to a 3 liter culture at hour 12 of a circadian-entrainment schedule (C) for 1 hour prior to the harvest by centrifugation. This stock is designated here (1 h) ^{3}H-Ur RNA, and is the same stock employed in a previous study.[7] Its specific activity is 2360 cpm/microgram.

31 hour pulse, 5 hour chase: 10 mCi of 5-^{3}H-uridine (NEN) in 10 ml of water was added to a 3 liter culture in the ultradian mode(U), and cells continued to divide in the U mode for 31 hours. The cells were centrifuged out of the labeled medium in a Sorvall GSA rotor (9000 rpm, 10 min.) and resuspended in fresh culture medium, and returned to the shaker for 5 hours, after which the harvest began. This RNA stock is designated (31 h)Ur RNA, and is the same stock used in previous work.[7]

RNA preparation: RNA was prepared from Tetrahymena cells by the methods previously outlined.[7] The RNA stock (31 h)Ur RNA was fractionated by sucrose gradient centrifugation to obtain a ribosomal-enriched cut, the "cut-3" of a previous report.[7] Its specific activity is 3000 cpm/microgram. This ribosomal-enriched cut of long-term labeled RNA is the one used in the hybridization experiments herein with the stock designation, (31 h) r RNA.

ERRF and SRF DNA preparation: Tetrahymena DNA was prepared as previously de-

scribed,[7] and dissolved in 0.2 X SSC (SSC = 0.15 M NaCl + 0.015 M sodium citrate) and sheared in a Ribi Cell fractionator (Sorvall, Inc.) at 50 kpsi at 10°C. Electron microscopic measurement of fragment lengths on other DNA preparations treated in this way yield pieces with an average molecular weight of 750,000 daltons. Sheared DNA was concentrated by passing it thru dry biogel P-100, and collecting the concentrate by vacuum filtration. DNA solutions at a final concentration of 450 µg/ml were heated in a water bath at 100°C for 5 minutes, brough to 0.12 M in phosphate buffer and incubated at 44°C to a $C_o t$ of approximately 100 ($C_o t$ is defined as the product of the initial DNA concentration and the time as expressed in units of Mol/liter X sec.). Under these conditions essentially all of the highly redundant DNA sequences of the Tetrahymena genome reassociate.[13] ERRF and SRF DNA components were separated by chromatography on hydroxylapatite,[15] and stored at -10°C until needed. A select elution profile for such a DNA preparation is shown in Figure 1. The amount of ERRF recovered from cells with a 30 hour generation time is 30% of the total DNA.

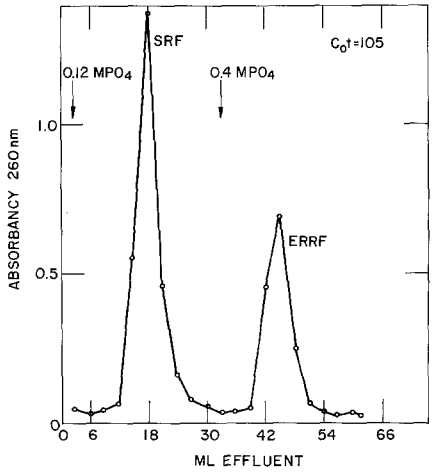

Fig. 1: Hydroxylapatite separation of the ERRF and SRF DNA components of *Tetrahymena* DNA from slowly dividing cultures. 9.2 O.D. units (260 nm) of denatured DNA in 2.0 ml was loaded on top of a 1.0 cm X 15 cm hydroxylapatite column maintained at 44°C after reassociation to a $C_o t$ of 105 (see Methods). Stepwise elution with first 0.12 M and then with 0.4 M phosphate buffers is indicated by the arrows. The effluent was monitored at 260 nm in a flow-cell assembly in a Gilford recording spectrophotometer. 1.5 ml fractions were collected and their absorbance at 260 nm plotted in the figure. SRF DNA was eluted off in the first peak by 0.12 M phosphate buffer, and the ERRF DNA was eluted off by the 0.4 M phosphate buffer step.

Preparation of DNA filters: The DNA was denatured in alkali, applied to nitrocellulose membrane filters (S & S, 25 mm, Sartorius, Division of Brinkman) dried and cut into quarters as previously described.[7] Experiments using ^{3}H-thymidine-labeled *Tetrahymena* DNA showed that when 5 µg of DNA was passed through the filter

(1.25 µg/quarter filter), 90% of unsheared DNA was retained throughout the hybridization, RNAsing, and washing procedures, while less than 40% of ERRF and 20% of SRF DNAs were so retained by filters. In these experiments, solutions with 40 µg of ERRF or SRF DNA were passed through the whole filter (10 µg/quarter filter).

DNA/RNA hybridization procedure: All experiments involve hybridization of RNA onto DNA bound on membrane filters. Ten quartered filters were annealed in various RNA concentrations in 33% formamide (Eastman Organic, New York) and 2 X SSC in 2.5 ml in 20 ml scintillation vials at 25°C. Other procedures for the hybridization assay, counting procedures, and statistical treatment of the hybridization data have been presented elsewhere.[7]

RESULTS: Kinetic curves for SRF and ERRF DNA with ribosomal-enriched RNA (31 h) rRNA, and with pulse-labeled RNA (1 h) ^{3}H-Ur RNA are shown in Figure 2. Reaction kinetics for SRF (Fig. 2a) are slow as seen by all curves showing the continual climb in counts with time. This is in contrast with the flatness of the curves against the ERRF DNA in Figure 2b. (Note the different scales.)

For ribosomal-enriched RNA (bottom curve in both plots) the major part of the hybridization occurs early in the reaction with ERRF DNA and additional counts

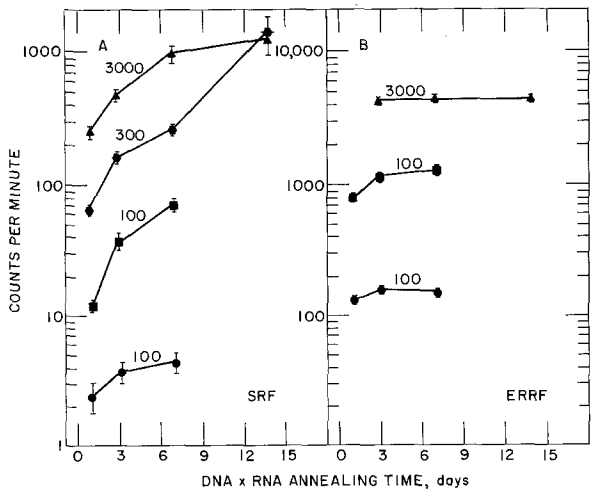

Fig. 2: Family of kinetic-hybridization curves with A, 10 µg of SRF DNA per filter, and B, with 10 µg of ERRF DNA per filter. SRF and ERRF DNA filters were annealed in 2.5 ml of FOSC and different concentrations of either one-hour pulse-labeled RNA (upper curves: ■—■, 100 µg/ml; ●—●, 300 µg/ml; and ▲—▲, 3000 µg/ml), or 31 hour pulse, 5 hour chased ribosomal-enriched RNA (bottom curve: ●—●, 100 µg/ml). Each point represents the mean with 2 SE for 10 filters. Mean counts on blank filters (5/ml) have been subtracted.

accrue slowly thereafter, while the hybridization of SRF DNA filters proceeds slowly throughout the time course of the reaction.

Kinetic curves for the same two RNA stocks against unfractionated DNA filters is shown in Figure 3. Again the rapid and slow components can be seen in the pulse-labeled RNA (upper three curves) the slow increase with time after the initial rapid gain. This is particularly marked when contrasted to the flatness of the curves versus the ERRF DNA in Figure 2b. (The drop in counts seen with the 3000 µg/ml after day 1 is not understood. Such exceptional points have been erratically observed in diverse hybridization experiments and defy analysis because they cannot be controlled or correlated with any one parameter and remain an enigma.) The long-lived-ribosomal-enriched RNA "plateaus" at a lower count as it did with ERRF DNA and note the overlap of the three curves for different concentrations of pulsed RNA.

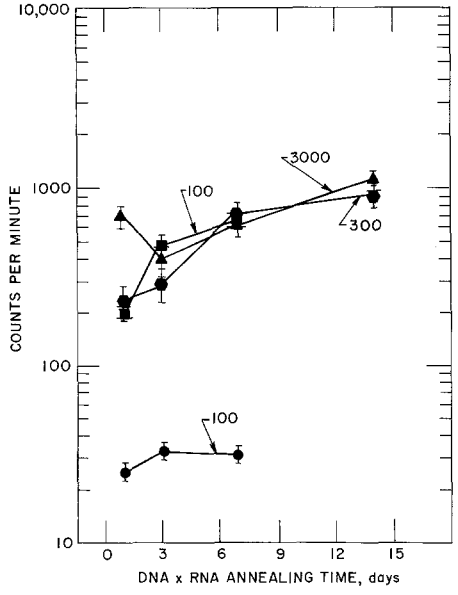

Fig. 3: Family of kinetic-hybridization curves with 1.25 µg of unfractionated *Tetrahymena* DNA per filter and different concentrations of either one-hour pulse-labeled RNA (upper three curves: ■—■, 100 µg/ml; ●—●, 300 µg/ml; and ▲—▲, 3000 µg/ml) or 31 hour pulse, 5 hour chased ribosomal-enriched RNA (bottom curve: ●—●, 100 µg/ml). Each point represents the mean with 2 SE for 10 filters. Mean counts on blank filters (5/ml) have been subtracted. Annealing conditions as given in legend of Figure 2.

DISCUSSION: In previous reports[9-11] hybridization of RNA to the non-repeated DNA of the eukaryotic genome was achieved without the physical separation of the nucleotide sequences involved, and were done in a liquid-liquid system. In the present study the disadvantages of competing DNA/DNA strand reassociation, and the problem of RNA degradation have been largely avoided by the use of low temperature annealing conditions,[7] and by the use of ERRF and SRF DNA immobilized to membrane filters. These advantages are somewhat offset by the reduced reten-

tion of sheared DNA on nitrocellulose filters (see Methods).

Nevertheless, the results presented above clearly show that the arbitrary separation of Tetrahymena DNA into ERRF and SRF DNA components is justified in terms of increased resoltuion of the DNA sites complementary to distinguishable RNAs in the given RNA stock with the kinetics of hybridization of SRF and ERRF DNA sequences being markedly different.

The hybridization results with DNA from a slowly growing culture unfractionated and fractionated into ERRF and SRF fractions with a $C_o t$ of 105 show that SRF DNA can serve as a template for RNA transcription. Since annealing ERRF DNA filters for 3 days with 100 µg/ml of RNA yields as many counts as 3 to 30 X that RNA concentration versus SRF DNA filters annealed for 15 days, the contribution of SRF templates to total counts observed in a hybridization reaction is minor. Using the DNA fractionation technique is an effective method to study these less repetitive templates. The results also show that the templates for long-lived-ribosomal-enriched RNAs fall in the ERRF fraction but that these ribosomal-like templates, however, make up a minor class of the ERRF or total DNA since the curves plateau at much lower counts than an unfractionated pulse-labeled RNA.

If one assumes that the radioactivity in the ribosomal-cut of the labeled-chased RNA (sp. act. = 3000 cpm/µg) reflects predominately labeled ribosomal RNA molecules, and that saturation has occurred (Fig. 2b), and allowing for 40% retention and one strand of the DNA serving as template in transcription, then 2.5% of the ERRF DNA is complementary to rRNA. Using as the DNA content of the Tetrahymena cell ($1{:}5 \times 10^{-11}$ g.),[13] and the combined molecular weights of the two ribosomal RNAs (2×10^6 daltons),[16] this corresponds to approximately 1×10^5 rRNA cistrons per cell.

In a pulse-labeled RNA than the transcripts of the less repeated sequences and the ribosomal sequences even taken together are minority (20%) contributors to the total counts seen. The identity and physiological role (if any) of these non-ribosomal RNA transcripts from the more reiterated DNA sequences is at present a mystery. Perhaps, their occurrence relate to the family of genes expected on the hypothesis that the DNA of all eukaryotic cells is organized into many polycistronic replicons, i.e., the genome is multi-repliconic,[17] each of which possesses a large overlap of evolutionarily-derived homologous genes.

The observation that when less DNA was present on the filter (Fig. 3) the three different concentrations of the pulse-labeled RNA clustered shows that "saturation" had occurred; while against ERRF DNA two different "plateaus" are seen suggesting the presence of at least two different classes.

All this suggests that using different $C_o t$ values to fractionate the DNAs combined with different annealing conditions may be a fruitful approach to dissect

out of the complex hybridizing mixture pertinate classes of reactants and lead to a more intelligible interpretation of hybridization results.

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