Characterization of a RNA that accumulates only in non-dividing Tetrahymena thermophila

Stephen James Libby
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Zoology Commons

Recommended Citation
Libby, Stephen James, "Characterization of a RNA that accumulates only in non-dividing Tetrahymena thermophila " (1987).
Retrospective Theses and Dissertations. 9272.
https://lib.dr.iastate.edu/rtd/9272

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17" × 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6" × 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
Accessing the World's Information since 1938
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
Characterization of a RNA that accumulates only in non-dividing Tetrahymena thermophila

Libby, Stephen James, Ph.D.

Iowa State University, 1987
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages
2. Colored illustrations, paper or print
3. Photographs with dark background
4. Illustrations are poor copy
5. Pages with black marks, not original copy
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received
16. Other

__________________________________________________________
__________________________________________________________

U·M·I
Characterization of a RNA that accumulates only in non-dividing *Tetrahymena thermophila*

by

Stephen James Libby

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major: Zoology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1987
TABLE OF CONTENTS

INTRODUCTION 1
MATERIALS AND METHODS 5
RESULTS 15
DISCUSSION 74
LITERATURE CITED 83
ACKNOWLEDGMENTS 91
INTRODUCTION

The ciliated protozoan *Tetrahymena thermophila* is able to regulate its RNA and protein metabolism in response to a variety of normal environmental changes. One such response which has been reasonably well characterized is that exhibited by cells exposed to a hyperthermal stress or heat shock (Hallberg et al., 1984, 1985, and reviewed in Schlesinger et al., 1982). Following a thermal stress, ongoing RNA and protein synthesis is depressed, the genes encoding the heat shock proteins are specifically transcribed and non-heat shock mRNA are unloaded from polysomes, and are replaced with heat shock messages that become selectively translated. Temperatures of 37°C-42°C will elicit this response. Interestingly, the effects of temperature on cell division also are different at different temperatures although the heat shock response is similar at these different temperatures with respect to proteins synthesized. Subjecting cells to sub-lethal heat shocks is in fact a method employed to synchronize cell division since at 40°C or higher, cell division is arrested (Zeuthen and Rasmussen, 1972). After 1-1.5 hours at 40°C, cells will begin dividing
again albeit at a slower rate. At 41°C, cells can divide again after 4 hours but only once. At 42°C, cells do not divide at all unless returned to 40°C or lower.

Similar to the situation for heat shock, when growing cells are transferred to some starvation media, cell division is arrested immediately, the majority of mRNA is unloaded from polysomes, ribosome synthesis is substantially depressed, and total RNA content drops 30-50% (Hallberg and Bruns, 1976 and Calzone et al., 1983a). The dramatic metabolic affects of starvation are reversed by refeeding cells. Within 2 hours of refeeding, cells will resume cell division and enter log growth by 3 hours (Sutton and Hallberg, 1979). Clearly, heat shock and starvation represent two distinct physiological conditions which not only greatly affect cell division, but drastically and rapidly effect gene activity in *Tetrahymena thermophila*.

Heat shock and starvation are two conditions that have been shown to cause major changes in the pattern of genes expressed in *Tetrahymena*. Martindale and Bruns (1983) identified a RNA, called C5, that appeared to accumulate to a high level in starved cells. The
clone, pC5, was isolated from a cDNA library made from poly A+ RNA from starved, conjugating cells. Although this RNA was subsequently shown not to be conjugation specific, it did accumulate to be an abundant poly A+ RNA in starved cells. The interesting property of this RNA is that it accumulated in starved, non-dividing cells to be a very abundant RNA. If starved cells were refed and allowed to resume division, the level of C5 RNA fell quickly as the cells began dividing. This RNA also accumulated in heat shocked cells, which also cause cell division to become arrested. At temperatures which allowed cell division to continue, the level of pC5 RNA was greatly reduced as the cells began dividing. Heat shock conditions that prevented cell division from continuing also stimulated C5 RNA to be accumulated and maintained at a high level. The initial observation was that C5 RNA accumulated only during conditions that caused cell division to become arrested. More importantly, C5 RNA is not present in growing cells. Because the accumulation of this RNA appeared to be correlated with the cell division cycle, we chose to study in more detail how this RNA is regulated and what are the signals that cause its induction. In this study, we have measured the
accumulation of C5 RNA during conditions that are known to affect cell division. We have examined the accumulation of C5 RNA in growing, heat shocked, starved, and stationary cells. We find, as others have, that in growing cells, the level of C5 RNA is not detectable, but conditions that arrest cell division also induce the rapid accumulation of C5 RNA. We have also measured the absolute concentration of C5 RNA in long starved cells and found it to comprise a significant fraction of the poly A+ RNA in the cell. We have found that all the C5 RNA is transcribed from a single gene. In attempts to determine what might be the signal that is responsible for the induction of C5 RNA in non-dividing cells, we have found that continued DNA replication is not required for C5 RNA to be accumulated. We have shown that C5 RNA accumulation and maintenance has an absolute requirement for continued proteins synthesis. Although C5 RNA has some characteristics of a mRNA, our analysis of the sequence data and polysome loading profiles, and accumulation in the presence of α-amanitin suggest that this RNA may not be a mRNA.
MATERIALS AND METHODS

Strains, culture, and conjugation conditions

Wild type BII or BIV (Cu335) was used for all experiments studying C5 RNA metabolism. T. elliotti, tropicalis, canadesis, australis, and pyriformis were kindly supplied by Dr. David Nanney and E.B. Meyers, Univ. of II., Urbana, Ill. B*, Ag, BIII, and BII were supplied by Dr. Paul Doerder, Cleveland State Univ., Cleveland, OH. Cells were grown in 1% proteous peptone with 0.003% Sequesterene (CIBA-GEIGY) at 30°C. For all experiments, early logarithmically growing cell, less than 100,000 cells/ml were used. Starvation was accomplished by harvesting the cells by centrifugation at 250 x g and gently resuspending the pellet in 10 mM Tris-HCL (pH 7.5). Cells were resuspended at a density of 150,000-500,000 cell/ml. To refeed starved cells, a one-tenth culture volume of 10% proteous peptone was added and 1% proteous peptone added to dilute the cells to a concentration of between 50,000 and 100,000 cell/ml. Cells were heat shocked by placing a growing culture in a shaking water bath at the appropriate temperature. The temperature in the flask generally reached the temperature of the water bath in less than 3 minutes. To initiate conjugation of BII and BIII
cell were first starved for 12 hour 10 mM Tris-HCl pH 7.5 at 300000 cell/ml, then were mixed in equal cell numbers such that there was 6 x 10^6 cells total. The mixed cells were placed at 30°C and not moved for 3 hours. Within 30-45 minutes, greater than 90% of the cells had formed pairs. These cells were then processed for polysome preparation as described below.

Enzymes and chemicals

Generally, all chemicals were purchased from either Sigma or Fisher. Restriction enzymes, RNASin, and DNA ligase was purchased from Promega Biotec, Madison, WI. and New England Biolabs, Beverly, MA. Klenow, calf alkaline phosphatase, and aphidicolin were purchased from Boehringer Mannheim. T7 and T3 RNA Polymerase were purchased from Stratagene, San Diego, CA. The dNTPs and rNTPs were purchased from Pharmacia, dissolved in sterile 50 mM Tris base, and pH adjusted to 7.0 with NaOH. The dideoxy NTPs were purchased from Bethesda Research Labs, MD.

RNA extraction, electrophoresis, and hybridization

Total cellular RNA or RNA from polysome fractions was extracted and analyzed by Northern or slot blot hybridization as previously described with no changes (Hallberg et al., 1984 and Hallberg et al., 1985).
Either nylon (Biotrans, ICN, Irvine, CA) or Genetran (Plasco, Woben, MA) membranes were used for RNA transfer and slot blots. DNA transfers were done exactly as described in (Maniatis et al., 1982).

**Polysome preparations**

For polysome analysis, 1-2 x 10⁷ cells were collected by centrifugation at 250 x g, concentrated into one tube by centrifugation then resuspended in 4-5 milliliters of ice cold 30mM Tris-HCL pH 7.0, 20mM KAc, 30mM MgCl₂, 2% spermidine, and 2mM dithiothreitol (polysome lysis buffer) as quickly as possible. The cells were disrupted by using a Portable Homogenizer (Fisher) that had been washed and precooled with ice cold diethlypyrocarbonate (DEPC) treated water. Log or heat shocked cells could be disrupted with one pass through the press, while starved cells required two passes through the press to completely lyse the cells. The resulting lysate was centrifuged at 10,000g at 4°C for 10 minutes. The absorbance at 260 nm was determined and 10-15 A₂₆₀ units (1-1.5 mls) of the post mitochondrial supernatant were layered on 15 ml 15-50% linear sucrose gradients containing 25mM Tris-HCl pH 7.4, 10mM KCl, and 25mM MgCl₂, and centrifuged at 27,000 rpm at 4°C in a SW27.1 (Beckman) for 3.75 hours.
Gradients were collected in 16 one milliliter fractions and the absorbance read at 260 nm. RNA from each fraction was extracted as described above.

**Plasmids, cloning, and sequencing**

The description of plasmids containing the HSP 70 gene (encoding the heat shock 70 kD protein), pC6, pCl, and pC2 can be found in Hallberg et al. (1984) and Martindale and Bruns (1983). All plasmids were purified by alkaline lysis and banded on CsCl gradients (Maniatis et al., 1982). The plasmid pBS-A4 was constructed by ligating a 590 bp Aha III fragment from pDP6 (Pederson et al., 1984) containing a single 5s rRNA gene into the Sma I site of pUC9 (Messing, 1983) and transforming E. coli 7118 (Yanisch-Perron, 1985) resulting in the intermediate plasmid called pSL-A4. This plasmid was digested with Eco RI and Hind III, gel purified, and ligating into the Eco RI-Hind III site of the transcription vector, Blue Scribe (pBS) (Stratagene, San Diego, CA.) to result in the plasmid called pBS-A4. The plasmid (pBS-LC5.7) used for in vitro transcription was constructed by first cloning the 1.030 kb Pst I fragment from pC5.5 into the phosphatase Pst I site of pUC9. This plasmid was then digested with Bam HI and Hind III, gel purified by
electroelution, and digested with Hinf I to yield two fragments of 740 and 290 bp. The ends of these fragments were made flush, using Klenow, and then ligated into the Sma I site of pUC9. The plasmid containing the 740 bp insert, pLC5.7, was digested with Eco RI and Hind III, gel purified in low melting point agarose (SeaPlaque, FMC Colloids), and ligated into the Eco RI-Hind III sites of pBS using the protocol described in (Struhl, 1985). The pC5 series of cDNA clones were all cloned into the Pst I site of pBS and the resulting clones, called pBS-C5.1-6, were isolated, size of inserts verified by mini-preparations (Riggs and McLachlan, 1986), and purifieds described above. To sequence the clones, either commercially purchased primers to the T3 and T7 promoters were used or custom primers were synthesized and purified by the Nucleic Acid Facility, Iowa State University. The Sanger dideoxy chain termination method (Sanger et al., 1977) was used to sequence supercoiled plasmids according to Chen and Cesareni (1985). The sequencing reactions were separated on either 6% or 7% acrylamide, 8 M urea gels run in 89mM Tris-borate, pH 8.3 and 2mM EDTA. The gels were pre-run for .5 to 1 hours and the samples loaded and ran for 2.5 hours at 1700-2000 volts, then
loaded again and run for additional 2-2.5 hours. After electrophoresis, the gels were soaked in 10% methanol and 10% acetic acid for 10-15 minutes before being dried down onto Whatman 3MM paper under vacuum and heat. The dried gel was exposed to X-ray film (XAR-5, Kodak) for 12-24 hours at -70°C. Sequences were manipulated using the Beckman MICROGENIE computer program.

In vitro transcription

To synthesize sense and antisense RNA, pBS-LC5.7 was linearized with either Eco RI or Hind III to transcribe RNA from the T3 or T7 RNA Polymerase promoter in the presence of \( \alpha-{\text{P}}^{32} \text{ATP} \) (NEN) and cold rNTPs (Pharmacia) according to the manufacture's recommendations (Stratagene, San Diego, CA and Promega Biotec, Madison, WI). Radioactive RNA was separated from unincorporated nucleotides using spun column chromatography (Maniatis et al., 1982), ethanol precipitated, resuspended in 150 ul of DEPC treated water, and A260 read to determine concentration in ug/ml. To determine the amount of \( \alpha-{\text{P}}^{32} \text{P} \text{ATP} \) incorporated, 2 ul of sample was added to 400 ul of H2O and 10% TCA, precipitated on ice for 15 minutes,
trapped on glass fiber filters, and counted. The amount of RNA in vitro transcribed was about 15 ug from the T3 promoter and about 25 ug from the T7 promoter with an average specific activity $1 \times 10^4$ cpm/ug.

**RNA-RNA solution hybridizations**

To determine the coding and non-coding strands of pBS-LC5.7, RNA made from the T3 and T7 promoters were hybridized separately to duplicate Northerns. RNA made from the T7 promoter hybridized to the same C5 RNA band detected by nick translated plasmid pBS-LC5.7, while RNA from the T3 promoter did not hybridize to any RNA on the filter. The hybridization and RNAase A and T-1 (Sigma) conditions were done as described in (Lee et al., 1986 and Davidson, 1986) except that the length hybridizations were carried out for 40-48 hours in a volume of 40-45 ul. To calculate the concentration of C5 RNA per cell using long starved cells, we used the method described in Davidson (1986) and Lee et al. (1986). The slope of the linear portion of the titration curve in Figure 3 was determined by least squares estimate. From the slope, the concentration of C5 RNA in pg/cell was calculated as follows: ($\text{Slope in cpm/ug}$)($\text{Mass RNA per cell}$) / ($\text{Specific activity of the}$
probe) (Fraction of mature message represented by probe).

Quantitation of slot blots

Slot blot filters were exposed to pre-flashed X-ray film (X-AR5, Kodak,) according to Laskey and Mills (1975). The autoradiograms were scanned with a LKB laser densitometer and areas calculated using a HP-9874A digitizer coupled to a HP-85 computer to determine the relative concentrations of C5 RNA in either polysomal fractions or total cell RNA preparations from log, starved, refed, heat shocked, or conjugating cells.

DNA synthesis inhibition

Aphidicolin (Boehringer, Mannheim, a generous gift from R. Benbow) was used to inhibit DNA synthesis and thus cell division in Tetrahymena. Aphidicolin, dissolved in 50% DMSO, 50% ethanol, at 10 ug/ml was the lowest concentration needed to completely inhibit cell division. Cells were able to recover from the effects of the drug and resume dividing when transferred to fresh growth media. To determine how much aphidicolin inhibited DNA synthesis, cells were labeled in the presence of 10 ug/ml of aphidicolin and 20 uCi/ml of $^3$H-thymidine (Amersham, Il). Duplicate 100 ul
aliquotes were removed over a 3 hour period, lysed in 100ul of 0.1M NaOH, TCA precipitated on ice, filtered, and counted. Aphidicolin inhibited more than 90% the incorporation of TCA precipitable H^-thymidine counts greater in cells treated at 10 ug/ml. To determine what effect aphidicolin might had on protein synthesis, cells were pulse labeled for 10 minutes with ^H-lysine (Amersham, Il.) at 10 uCi/ml over a three-hour period. Duplicate 100 ul aliquotes of cells were lysed in 0.1 M NaOH, TCA precipitated, filtered, and counted. The ^H-lysine incorporation in aphidicolin treated cells was 85-90% of non-treated cells.

DNA extraction from Tetrahymena cells

Cells were harvested by centrifugation, washed with H₂O, and lysed by resuspension then in 10 mls of 0.5M EDTA, 10mM Tris-HCl pH 9.5, and 1% SDS (lysis buffer). Cells were then placed at 50°C for 20 minutes before the addition of 5 mls of lysis buffer containing 4 mg/ml pronase that had been pre-digested for 2 hours at 50°C was added. Another 5 mls of the pronase-lysis buffer mix were added to the lysate and the mixture was allowed to incubate overnight. A 1.5 ml of 1M NaCl per ml of original packed cell volume is added to the lysate and placed on ice for at least 5 hours. The
lysate is centrifuged at 10,000 rpm, 4°C for 20 minutes
and the supernatant is dialyzed overnight against a
large volume of 10mM Tris-HCl pH 8.0, 1mM EDTA (TE).
The DNA was precipitated by adding 2 volumes of cold
95% ethanol and one-tenth volume of 3M NaAc pH 5.2 and
placed at -20°C for at least 2 hours. The DNA was
pelleted, resuspended in TE, phenol-chloroform
extracted, and ethanol precipitated. The DNA was
pelleted and resuspended in TE and the concentration
determined by reading the absorbance at 260nm.
RESULTS

Accumulation of C5 RNA during starvation, refeeding, heat shock, and stationary growth

The description of the cloning and preliminary characterization of pC5 and the RNA transcript homologous to it is described in Martindale and Bruns (1983). The pC5.5 clone is a cDNA clone that has a 1.03 kb insert that has been shown to hybridize to a single RNA that is 1450 bases long in both starved as well as heat shocked cells (Hallberg et al., 1984). Because pC5 was isolated from a cDNA library made from poly A+ RNA of conjugating cells (which had been starved to induce this state), we first measured the kinetics of accumulation of the pC5.5 related transcript (or C5-RNA) during starvation. To do this, logarithmically growing cells were washed into starvation media and at various times during starvation, total RNA was extracted and analyzed by either Northern or by slot blot hybridization (see Materials and Methods). The results of one such analysis are shown in Figures 1 and 2. Following the initiation of starvation, C5 RNA accumulates rapidly from non-detectable levels in log cells (see below) and reaches a maximum level by 6 hours, remaining at this
Figure 1. Quantitation of C5 RNA in starved cells

Log cells were washed into 10 mM Tris (pH 7.5) for up to 24 hours and RNA was extracted at the time indicated. (A) Total RNA from log and starved cells was separated on denaturing agarose gels, transferred to membranes, and probed with P\(^{32}\)-nick translated plasmid copies of pC5 and pBS-A4 (containing one 5s rRNA gene). Hybridization to the 5s rRNA serves as an internal control for the amount of RNA loaded per lane. (B) Slot blot hybridizations of total RNA from log and starved cells. Each slot represents a one-fourth dilution of the previous. A total of 3 ug of total RNA was diluted to 0.76 ug, 0.19 ug, and 0.05 ug. These autoradiograms were scanned with a laser densitometer and areas under the peaks determined using a digitizer.
<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A**

Starve

**B**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>C5</th>
<th>5s</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**5s**
Figure 2. Accumulation of C5 RNA during starvation and refeeding

The data from Figure 1 are plotted showing the accumulation of C5 RNA during starvation. In a separate experiment, long starved cells were refed as described in the Materials and Methods. At the times indicated, RNA was extracted from cells and the amount of C5 RNA accumulated determined as described in Figure 1. The amount of C5 RNA remaining is shown as a percent of the maximum level in long starved cells.
level for at least 24 hours (the latest time point taken during starvation). This experiment has been repeated at least 4 times giving essentially identical results each time. As we wished to directly compare the number of C5 transcripts in cells subjected to a variety of physiological conditions, it was first necessary to measure the absolute amount of C5 RNA in cells under one particular physiological state. Thus, in subsequent experiments, all values may be compared directly to this standard amount per cell. We chose to make these initial measurements in cells starved for 24 hours. This was done by carrying out RNA-RNA solution hybridizations using the method described in Lee et al. (1986) and also in Davidson (1986). To that end, we cloned a 740 base pair fragment of pC5 into the transcription vector pBS, which contains the T3 and T7 RNA Polymerase promoters so that RNA could be in vitro transcribed from both strands (see Materials and Methods and Figure 14 for a map of the pC5 series clones and constructs). RNA transcribed from the T3 promoter was anti-sense (i.e., was complementary to the cellular transcript) and was used in the solution hybridizations to generate the data shown in Figure 3. The sensitivity of the assay is such that we could
Figure 3. Determination of the concentration of C5 RNA in 23 hour starved cell by RNA-RNA hybridization.

Total RNA from 23 hour starved cells was hybridized to P32-labeled sense and anti-sense RNA made from the T3 and T7 RNA polymerase promoters from the plasmid pBS-LC5.7 (see Material and Methods). The RNA-RNA hybridization were then digested with RNAase A and T-1, TCA precipitated, and counted. The RNAase resistant cpm represents stable hybrids between the cellular copy of C5 and the anti-sense in vitro (T7) transcribed RNA. The concentration of starved RNA used was 1 to 100 µg, 150 µg, and 200 µg. With increasing amount of total RNA, the amount of RNAase resistant cpm increased linearly when the anti-sense C5 RNA was used (open squares) and reached saturation at around 50 µg of total RNA. When the same starved RNA was hybridized with sense C5 RNA and if log RNA was hybridized with either sense or anti sense RNA, no RNAase resistant cpm over background could be detected (closed squares). The slope of the linear portion of the curve was used to calculate the concentration of C5 RNA per cell.
detect 0.5 ng of RNAase resistant hybrids in 1 ug of RNA from long starved cells. Long starved cells contain 0.15 pg/cell of C5 RNA as compared to Histone H4 at 0.001 pg/cell or 150 times less than C5 (Yu and Gorovsky, 1986). We could neither detect any RNAase resistant cpm over background covering a 200 fold concentration range (1 to 200 ug of total cellular RNA from log cells) when RNA from log cells was used with the same T3 RNA nor could we detect any using T7 RNA (from the sense strand) with either RNA from starved or log cells in two separate and identical experiments. Using data from Calzone et al. (1983b), we then calculated that C5 RNA should constitute 7.6% of the poly A+ RNA in long starved cells and Histone H4 0.06%. This represents 200,000 molecules per cell of C5 RNA and 1,600 molecules of histone H4 per cell. The lower limit of detection of our RNA-RNA hybridization is such that we could detect an RNA that is at least 1000 copies per cell. Therefore, log cells have less than 1000 copies of C5 RNA and we define non-detectable as less than 1000 copies or 0.0008 pg/cell of RNA. In all subsequent measurements, we then used the level of C5 RNA in starved cells as a basis for comparison. This allowed us to convert values generated from slot blot
Table 1. Quantitation of C5 RNA in growing and long starved cells

<table>
<thead>
<tr>
<th>RNA</th>
<th>Cell</th>
<th>Pg/cell&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Molecules per cell&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Poly A&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>Log</td>
<td>0.0008</td>
<td>1000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Starved</td>
<td>0.15</td>
<td>200,000</td>
<td>7.6</td>
</tr>
<tr>
<td>Histone&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Log</td>
<td>0.022</td>
<td>40,000</td>
<td>0.002</td>
</tr>
<tr>
<td>H4</td>
<td>Starved</td>
<td>0.001</td>
<td>1600</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated as described in Davidson (1986); also see Materials and Methods.

<sup>b</sup>Converted pg/cell to molecules per cell for C5 RNA. The number of histone H4 molecules per cell are from Yu and Gorovsky (1986).

<sup>c</sup>In starved cells, 2 pg of total RNA is mRNA. In log cells, there is 16 pg of mRNA per cell.

<sup>d</sup>Since there were no RNAase resistant RNA-RNA hybrids protected using 200 ug of log RNA, the amount of C5 RNA in log cells must be less than 1000 molecules per cell.
dilution series of RNAs from cells under other physiological states into absolute values.

Starved cells are 100% viable in starvation media (10 mM Tris- HCl, pH 7.5) and will enter logarithmic growth after 2-3 hours when returned to growth medium (1% peptone and 0.003% Sequesterene) (Hallberg and Bruns, 1976). Consequently, we asked what happens to the level of C5 RNA following the refeeding of starved cells. The results of slot blot analysis of total RNA isolated from cells during the first 4 hours of refeeding are shown in Figure 2. By the first one hour of refeeding, the level of C5 RNA has dropped to less than 6% of the 23 hour starved level and by two hours, as the cells are beginning to divide and enter log growth, the level of C5 RNA is less than 1% of the long starved level (cell numbers not shown). These results show that even before cell division resumes in the culture of refeed cells, the level of C5 RNA decreases dramatically. Whether this decrease is simply the result of the cessation of production of C5 RNA or is also due to an increased rate of degradation of this RNA is not known.

Although the heat shock response is similar within the range of 39° to 42° with respect to the induction
Figure 4. Effect of temperature on cell division

Logarithmically growing cells were shifted to 39°-42°C for four hours. Duplicate samples were taken at the times indicated and counted using a Coulter Counter. The arrow denotes when the cells were heat shocked.
of heat shock proteins (Hallberg et al., 1985 and reviewed in Schlesinger et al., 1982) there is a pronounced temperature effect difference with respect to the cell division behavior of the treated cells. When cells are shifted from 30°C to 39°-40°C, further cell division ceases for about 90-120 minutes and then resumes. Cells shifted to 41°C will divide again but only once after 4-5 hours following the temperature shift. Cells shifted to 42°C do not divide at all but remain viable at this temperature for at least 24 hours. These cells will divide again if allowed to recover at 40°C or lower. The effect of temperature on cell division in *Tetrahymena* is shown in Figure 4.

From previous studies (Hallberg et al., 1984), we know that C5 RNA is induced when cells are heat shocked at 41°C. We therefore asked, does the level of C5 RNA increase with the severity of the thermal stress and does its appearance (or disappearance) in any way correlate with the cell division properties of the cell. To determine this, cells were heat shocked at 39°-42°C and RNA extracted at various times during a 4 hour period. The amount of C5 RNA accumulated was determined by quantitating slot blot hybridizations. Figure 5a shows the effect of heat shock on C5 RNA
Figure 5. Accumulation of C5 RNA during heat shock

RNA as extracted from heat shocked cells shown in Figure 4 and the amount of C5 RNA accumulated during the heat shock determined using slot blots as described in Figure 1 (also see Materials and Methods). (A) C5 RNA accumulation during heat shock at 39°C to 42°C for four hours. (B) The data from 42°C heat shock (A) including recovery at 30°C for three hours are plotted.
accumulation. At all temperatures, the level of C5 RNA rises rapidly and reaches a maximum by 1 hour. The level of C5 RNA accumulation at 39°C parallels that at 40°C even though division resumes at 39°C earlier than it does at 40°C. As cells begin dividing by 90-120 minutes at both 39°C and 40°C, C5 levels drop to about 20% of maximum and this level remains constant for at least 4 hours. When cells are heat shocked at 41°C, C5 RNA accumulates to a level 3.5 times that of 39°C or 40°C heat shocked cells and this level is maintained until 4 hours at which time the cells are able to complete only one round of division. The drop in the level of C5 RNA at about 4 hours at 41°C reflects the time when the cells are able to divide. When heat shocked at 42°C, the level of C5 RNA accumulated is 9.1 times that of 40°C and remains at this level until cells are allowed to recover at 30°C. During recovery, C5 RNA levels drop rapidly within the first 60 minutes and by 1.5-2 hours, when the cells have resumed division, there is less than 4% of the maximum level remaining (Figure 5b). The results of these experiments demonstrate a temperature effect on the level of C5 induction: the higher the temperature the greater the C5 RNA accumulation. With this in mind, we
Figure 6. Accumulation of C5 RNA at 43°C

Growing cells were shifted to 40°C for 4 hours until the cells had divided twice, then shifted to 43°C for 1 hour. The cells were then allowed to recover at 30°C for 3 hours. At the end of 3 hours, cells began to divide. Part of the culture was left at 43°C for 2 and 6 hours. During the time at 43°C, the cells did not divide and did not die. Total RNA was extracted at the times indicated and the amount of C5 RNA accumulated was determined as described in Figure 1.
asked if the level of C5 RNA could be superinduced by shifting cells to 43°C, a temperature shift that would normally be lethal without a prior heat shock (Hallberg et al., 1985). To do this experiment, cells were first heat shocked at 40°C for four hours until they had divided twice, then shifted to 43°C. Figure 6 shows that when cells are heat shocked at 42°C, C5 RNA accumulates only 3-5 times the level in 40°C heat shocked cells and remains at this level for at least 6 hours. When cells were allowed to recover at 30°C, C5 RNA levels dropped rapidly, just as in 42°C heat shocked cells, and was undetectable by 3 hours as the cells started dividing. A possible explanation for the lack of "superinducibility" of C5 RNA at 43°C is that the pre-heat shock may attenuate the response of C5 gene expression at 43°C.

Our data so far have shown that conditions such as heat shock and starvation that cause the arrest of cell division induce the accumulation of C5 RNA. We wished to examine the pattern of C5 RNA accumulation in cells that are entering stationary phase of growth, a condition in which cell division never actually stops, but slows considerably (Sutton and Hallberg, 1979; Reuter et al., 1980). The division rate for
Figure 7. Accumulation of C5 RNA in stationary cells

Total RNA was extracted from a culture of cells over a 37 hour period as they entered stationary growth. This RNA was separated on agarose gels, transferred to membranes, and probed with $^{32}$P-nick translated plasmid pC5. The lanes of the autoradiogram correspond to the cell numbers as follows: lane 1=44,000, lane 2=87,000, lane 3=137,000, lane 4=315,000, lane 5=678,000, lane 6=774,000, and lane 7=995,000 cells/ml. C5 RNA is detectable when the cells reach about 315,000 cell/ml and levels increase as cells become increasingly dense. The drop in the level of intensity of C5 RNA hybridization in lane 7 is due to underloading as judged by 5s rRNA hybridization (data not shown).
Tetrahymena is about 3 hours at 30°C up to a density of about 300,000 cells/ml, at which the division rate begins decreasing as cells become increasingly dense. To determine if and when C5 RNA accumulates in stationary or in late log cells, total RNA was extracted from cells during growth into plateau stage of growth and the accumulation of C5 RNA determined by Northern analysis as shown in Figure 7. C5 RNA first becomes detectable in cells at a density of 317,000 cells/ml and increases gradually from 678,000 cell/ml to 943,000 cells/ml (plateau). In a separate experiment (data not shown), when stationary cells were diluted back to 50,000 cells/ml, the level of C5 RNA had dropped below the level of detection by one hour. Relative to long starved cells, stationary cells accumulate 0.54 times the amount of C5 RNA. These results show that a change in cell division rate is a sufficient signal for induction of C5 RNA. The level of C5 RNA accumulated during starvation, heat shock, and stationary cells are summarized in Table 2.

**Signal for C5 induction**

The mechanism by which cell division is arrested when Tetrahymena cells are heat shocked or starved is not known and is probably not the same for each
Table 2. Summary of C5 RNA Levels Relative to Long Starved Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of Starved</th>
<th>Molecules per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>7.8</td>
<td>16,000</td>
</tr>
<tr>
<td>40</td>
<td>7.8</td>
<td>16,000</td>
</tr>
<tr>
<td>41</td>
<td>27.3</td>
<td>54,000</td>
</tr>
<tr>
<td>42</td>
<td>70.9</td>
<td>140,000</td>
</tr>
<tr>
<td>43</td>
<td>27.3</td>
<td>54,000</td>
</tr>
<tr>
<td>Plateau</td>
<td>54.5</td>
<td>108,000</td>
</tr>
<tr>
<td>Log</td>
<td></td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

*Determined by slot blot hybridization of the one hour time points shown in Figure 4 for heat shock and at 995,000 cell/ml for plateau. All RNAs were on the same filter and hybridized first with nick translated pC5. After autoradiography, the pC5 probe was removed by soaking the filter in boiling 0.1x SSC, 0.1% SDS. Filters were re-hybridized with the plasmid containing the 5s gene to normalize for the amount of RNA loaded per slot between samples. Autoradiograms were scanned and quantitated as described in Materials and Methods.
condition. However, in both cases, DNA replication is interrupted and it also slows considerably in stationary cells (Anderson, 1972, Reuter et al., 1980, Mowat et al., 1974, and Zeuthen and Rassmussen, 1972). We therefore wished to determine if the signal for C5 induction is somehow coupled to DNA replication. This was accomplished by blocking DNA replication in growing cells with aphidicolin and asking if C5 RNA accumulated. Unlike many DNA polymerase inhibitors with a multitude of side effects, aphidicolin is a specific inhibitor of DNA polymerase a (reviewed in Huberman, 1981). An important characteristic of this inhibitor is that it does not inhibit protein synthesis (Ikegami et al., 1978) or RNA transcription (Nishimura et al., 1979). The highest concentration of aphidicolin required to completely arrest cell division in Tetrahymena was found to be 10 μg/ml. At this concentration of aphidicolin, DNA synthesis is inhibited more than 90%, while protein synthesis continues at 91% at the level of untreated cells (see Materials and Methods). The results in Figure 8a show that no C5 RNA accumulated during the 4 hours the cells were incubated with the drug even though cell division was completely arrested. We found the same results
when the experiment was repeated with growing cells. We found later that protein synthesis is absolutely required for the induction and maintenance of C5 RNA (see below). We could not rule out the possibility that aphidicolin might be interfering with some other component of cellular metabolism not detected by measuring at total \(^3\)H-lysine or thymidine incorporation, preventing C5 RNA from being accumulated. To test this possibility, cells were heat shocked in the presence or absence of aphidicolin at 40° C to determine if C5 RNA accumulation is altered in the presence of the drug. These results also shown in Figure 8a indicate that during the 40°C heat shock in the presence of aphidicolin at 10 µg/ml, C5 RNA metabolism is essentially identical to that in 40°C heat shocked non-treated cells. Thus, aphidicolin has no side effect which prevents C5 RNA induction from occurring. It simply does not have the property of inducing it on its own.

Not only does the regulation of C5 RNA metabolism during refeeding occur in the absence of cell division, but it also occurs in the absence of DNA replication (Rudick and Cameron, 1972). The level of C5 RNA drops rapidly during refeeding (Figure 1) in the absence of
Figure 8. Effect of aphidicolin on C5 RNA induction at 30°C, 40°C, and during refeeding

(A) To determine if inhibiting DNA synthesis will cause C5 RNA to accumulate, cells at 30°C were treated with aphidicolin at 10 μg/ml for 30 minutes (lane b), 60 minutes (lane e), 120 minutes (lane l), and 240 minutes (lane m). In lanes a, h, and l, is RNA from non-treated cells at 30, 60, and 240 minutes at 30°C. In the non-treated cells as well as the treated cell, no C5 RNA accumulated. This autoradiogram is overexposed to see if any C5 RNA was accumulated. On the same filter is shown the results of heat shocking cells in the presence of aphidicolin to determine if the drug interferes with the normal metabolism of C5 RNA during heat shock. Cells were heat shock at 40°C in 10 μg/ml aphidicolin for 30 minutes (lane d), 60 minutes (lane g), 120 minutes (lane k), and 240 minutes (lane o). Lanes c, f, j, n, are RNA from non-treated cells at 30, 60, 120, and 240 minutes of heat shock. Lane p is RNA from 90 minute starved heat shocked cell that served as a positive control for C5 RNA induction and HSP 70 induction. This filter was probed with nick translated pC5.5, the plasmid clone for HSP70, and pBS-A4 (5s gene). (B) Sixteen hour starved cells were re-fed in the presence of aphidicolin at 10 μg/ml for 30 minutes (lane 3), 60 minutes (lane 5), 120 minutes (lane 7) and 3.5 hours (lane 9). RNA from non-treated cells at the same times are shown in lanes 2, 4, 6, and 8. Lane 1 is RNA from non-treated 16 hour starved cells.
DNA synthesis, which does not begin until about 3 hours into refeeding. We wanted to determine if this decline could be prevented by inhibiting DNA replication during refeeding. To do this, we refed cells in the presence of 10 ug/ml aphidicolin and extracted total RNA from the cells at various times until the non-treated cells had resumed division. The results of this experiment, as shown in Figure 8b, show that aphidicolin had no effect on the decline of the level of C5 RNA during refeeding. In the presence of the drug, cells did not divide until transferred to fresh medium. Just as in heat shocked cells, the regulation of C5 RNA proceeds in the absence of cell division even when DNA replication is blocked. From these experiments, we conclude that the primary signal for C5 RNA regulation is not directly coupled in some regulatory fashion to DNA replication. We further conclude that the inhibition of cell division may be a necessary condition, but is certainly not a sufficient condition to induce C5 accumulation.

Although we have not been able to dissect the mechanism that is responsible for the induction of C5 RNA, we have determined some other conditions that are required for its accumulation and maintenance. This
was done by determining the effect of the protein synthesis inhibitor cycloheximide on C5 RNA accumulation and maintenance. It has been shown that some mRNAs are either stabilized or even superinduced in the presence of protein synthesis inhibitors such as cycloheximide (Linzer and Wilder 1987, Linial et al., 1985, Slve et al., 1984, and Stimac et al., 1984). We tested the effects of two different concentrations of cycloheximide on the accumulation and maintenance of C5 RNA in fresh and long starved cells. One concentration (5.0 ug/ml) completely and permanently inhibits any further protein synthesis. The other (0.5 ug/ml) has a transient effect, initially protein synthesis is completely inhibited, but gradually cells "adapt" and resume protein synthesis. During starvation in the presence of cycloheximide, the accumulation of C5 is delayed for two hours at both 0.5 and 5.0 ug/ml, then begins to accumulate in cells treated with 0.5 ug/ml by 2 hours and eventually reaches 89% the level in non-treated cells by 6 hours, but not at 5.0 ug/ml. The level of C5 RNA reaches only about 2% that of non-treated cells by 6 hours in the presence of 5.0 ug/ml cycloheximide (Figure 9a). When 23 hour starved cells were treated with either concentration of
cycloheximide, the level of C5 RNA dropped rapidly at both concentrations, but as in fresh starved cells, C5 RNA began to accumulate in cells treated with 0.5 µg/ml of cycloheximide by 2 hours and by 6 hours reached 80% the level of C5 RNA in non-treated cells. C5 RNA in cells treated with 5.0 µg/ml does not accumulate to appreciable levels by 6 hours (Figure 9b). The delay in the appearance of C5 in fresh starved cells or the drop then recovery of C5 in long starved cells reflected the time required for the cells to adapt to cycloheximide (Sutton et al., 1979 and Frankel, 1970). We repeated these experiments with 15 minute and 90 minute 41°C heat shocked cells to determine if accumulation and maintenance of C5 RNA during heat shock is sensitive to cycloheximide as in starved cells. Cycloheximide had the same effect on the accumulation and maintenance of C5 RNA in heat shocked cells as in starvation (data not shown).

Was the response of C5 RNA to cycloheximide specific for C5 RNA or was it common to other or all mRNAs of *Tetrahymena*? We tested this by re-probing filters containing RNA from the previous experiments with a cDNA clone for a mRNA encoding a putative surface antigen (manuscript in preparation) called pC6
Figure 9. Effect of cycloheximide on C5 RNA accumulation and maintenance in starved cells

(A) Effect of cycloheximide on C5 RNA accumulation in fresh starved cells. Growing cells were washed into 10 mM Tris (pH 7.5) containing no drug (open squares), 0.5 ug/ml (closed diamonds), or 5.0 ug/ml of cycloheximide (closed squares). RNA was extracted at the times indicated and the amount of C5 RNA accumulated determined as in Figure 1. (B) Effect of cycloheximide on C5 RNA maintenance in long starved cells. Long starved cells were either non-treated (open squares), treated with 0.5 ug/ml (closed diamonds), or 5.0 ug/ml of cycloheximide (closed squares) for 6 hours, RNA extracted at the times indicated, and the amount of C5 RNA determined as in Figure 1. (C) Northern analysis of the RNA from (A) probed with pC6. pC6 is a clone for a putative surface antigen and the autoradiogram shows its concentration does not change as did C5 RNA in the same cells.
A

RELATIVE GROWTH

0 40 80 120 160 200 240

MINUTES

CONTROL

0.5 UR/ML

5.0 UR/ML

B

RELATIVE GROWTH

0 100 200 300

MINUTES

CONTROL

0.5 UR/ML

5.0 UR/ML

C

log starve + cyc

C6
(Martindale and Bruns, 1983). Cycloheximide had no effect on the concentration of this RNA and an example of the re-probed Northern of fresh starved cells (from the experiment shown in Figure 9a) treated with cycloheximide and is shown in Figure 9c. We have reprobed slots blots of RNA from long starved cells treated with cycloheximide and the level of pC6 RNA does not change (data not shown). These results demonstrate a unique dependency on continued protein synthesis for C5 RNA to be accumulated and maintained. This phenomenon appears to be a unique feature of C5 RNA regulation with respect to other mRNAs of Tetrahymena.

Polysomal distribution of C5

C5 RNA is a poly A+ RNA 1450 bases long and could potentially encode a peptide of 45-50 KD. However, analysis of 1 and 2 dimensional protein gels from starved, heat shocked, and stationary cells did not detect a peptide in this size range present in an amount proportional relative to the amount of C5 RNA (data not shown). A possible explanation for this finding is that C5 RNA (if it truly is a mRNA) is utilized only during a specific time during the physiological conditions that induce its accumulation.
To explore this possibility, we isolated polysomes from starved, heat shocked, and conjugating cells and determined the distribution of C5 RNA in polysome gradients. Cells were shifted to a particular physiological condition, harvested, mechanically disrupted, and the post mitochondrial supernatant was then layered onto a 15-50% sucrose gradient and centrifuged. RNA from each fraction was extracted and the amount of C5 RNA in the fractions determined by scanning slot blots. The results of polysome preparations from starved and heat shocked cells are shown in Figure 10. By 1.5 hours of starvation (Figure 10a), 12% of C5 RNA is found in the polysomal region of the gradient (fractions 8-16) with the majority of C5 RNA sedimenting with or above the monosomes. By 6 hours of starvation (Figure 10b), 24% of C5 RNA is polysomal and in 23 hour starved cell (Figure 10c), 34% of the total C5 RNA loaded onto the gradient can be found in the polysomal region. Polysomes prepared from 90 minute 40°C heat shocked cells (Figure 10e), showed 25% of C5 RNA sedimenting in the polysome region. Although during starvation, more C5 RNA become loaded onto polysomes, it never becomes loaded to a degree that one might expect for a poly A+ RNA with a
Figure 10. Polysomal distribution of C5 RNA during starvation and heat shock

Panel A, 1.5 hour starved, panel B, 6 hour starved, panel D, 23 hour starved, and panel E, polysomes from 90 minute 40°C heat shocked cells. In panel C is shown the A$_{260}$ profile of the fraction from log and 23 hour starved polysome preparations. Polysomes were prepared from cells that were starved for 1.5, 6, and 23 hours or heat shocked at 40°C for 90 minutes. RNA from each fraction prepared for slot blot hybridization analysis as in Figure 1 and probed with nick translated pC5.5. The resulting autoradiograms were scanned and are plotted as the relative amount of C5 RNA found in each fraction of the gradient. The direction of sedimentation is from left to right. Fraction 1 represents the top of the gradient and fraction 16 the bottom. In panel F is shown the Northern transfers from each of the gradients probed with pC6 to show the location of a loaded, utilized mRNA. Also shown is the Northern of 23 hour starved polysome probed with pC5 and the 5s rRNA containing plasmid to show the distribution of C5 RNA in the gradient by autoradiography. The location of the monosome and polysomes can be seen by the location of the hybridization to 5s rRNA.
concentration as relatively high as that of C5. To show that our polysome preparations are in fact separating polysomal from non-polysomal messages, Northernns from all gradients were re-probed with pC6 (1470 bases) (and in some cases a probe for hsp 73) as shown in Figure 10f. The majority of pC6 RNA (and hsp 73 mRNA when present) sedimented in the polysomal region. This sedimentation behavior is expected since the surface antigen is expressed during starvation.

One possible explanation for the loading pattern in starved and heat shocked cells is that the signal(s) for C5 RNA accumulation and utilization are not the same. Since C5 was initially isolated from conjugating cells, it is possible that C5 is utilized during conjugation. Martindale and Bruns (1983) and others (Ron and Suhr-Jessen, 1981) have shown there is a peak of protein synthesis about 3 hours into conjugation and have identified the synthesis of the conjugation specific peptides. To determine if C5 is loaded maximally during conjugation, we prepared polysomes from conjugating BII and BIII cells that had been mixed and conjugating for 3 hours. As a control, we also prepared polysomes from separately starved BII and BIII cells that were mixed just prior to lysis. RNA from
Figure 11. Polysomes from non-conjugating and conjugating cells

Polysomes from conjugating and non-conjugating cells were prepared to determine if C5 RNA is loaded during conjugation. (B) Mating type BII and BIII were separately starved for 12 hours, then mixed in equal concentrations and allowed to sit and form pairs for 3 hours. At the end of this time, greater than 90% of the cells had formed pairs. The cells were then harvested and polysomes prepared as in Figure 9. As a control, BII and BIII were mixed at the same cell concentration just prior to harvesting. (A) RNA from each gradient fraction (non-conjugating and conjugating) was separated by gel electrophoresis, transferred to membranes, and both filters probed with nick translated pC1, pC2, pC5.5, and pBS-A4. pC1 and pC2 hybridize to conjugation specific RNAs. The pC1 transcript is 2800 bases and the pC2 transcript is 7540 bases long. The intensities of hybridization are not equal and reflect the differences in specific activity of the probes. The hybridization to the 5s rRNA shows the location of the monosome and polyribosomes. The direction of sedimentation is from left to right. The first lane of the autoradiogram is total RNA and represent what was loaded onto the gradients.
these fractions was transferred to nylon membranes and both filters were probed with pC5 and two conjugation induced RNAs pC1 and pC2. The autoradiogram from this experiment (Figure 11a-b) shows no real difference in the amount of C5 RNA loaded as compared to non-conjugating cells. However, C1 and C2 RNAs, which are 2800 bases and 7540 bases respectively, were induced and loaded onto polysomes only in conjugating cells. We have not been able to find a condition where the majority of C5 is loaded onto polysomes, but since we have not isolated polysomes from stationary cells or different times into conjugation, it is still possible that there might be a condition that causes C5 RNA to become fully loaded. Alternatively, the amount of C5 RNA that is polysomal in long starved cells may be all that is ever loaded.

**C5 RNA may not be transcribed by RNA Polymearase II**

Although we have not been able to find a peptide of the size that might be encoded by C5, it nonetheless has characteristics that indicate that it could be an mRNA, such as being cloned as a cDNA and loaded onto polysomes, albeit not completely. Another method to show whether C5 may in fact be a mRNA is to determine
Figure 12. The accumulation of C5 RNA is not inhibited by α-amanitin

Total RNA was extracted from cells incubated for 40 minutes at 30°C in 10 mM Tris, pH 7.5 (lane a), RNA from cells that had been starved for 30 minutes, then heat shocked at 40°C for 30 minutes with no drug, and from cells that were incubated for 10 minutes at 30°C in 10 mM Tris (pH 7.5) containing α-amanitin at 150 ug/ml (lane C), 300 ug/ml (lane D), or 600 ug/ml (lane E) and the shifted to 40°C for an additional 30 minutes with α-amanitin still present. The RNA from these cells was extracted, electrophoresed, transferred to membrane, and probed with P32-nick translated plasmid copies of HSP 70, pc5.5, and pG8 (a RNA polymerase III transcript). The amount of C5 RNA which accumulated in cells treated with 600 ug/ml α-amanitin was 30-50% of non-treated cells. The level of HSP 70 at the same concentration was 15-20%. The amount of G8 RNA at 600 ug/ml was 80-85% of non-treated cells. (This figure is taken from Kraus, Good, and Hallberg, 1987.)
<table>
<thead>
<tr>
<th>30'</th>
<th>40'</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
</tr>
</tbody>
</table>

- hsp73
- C5
- pG8
if its synthesis is inhibited by the RNA polymerase II inhibitor, α-amanitin. All mRNAs are transcribed by RNA polymerase II and their synthesis is therefore sensitive to the inhibitory effects of this drug. To determine if C5 RNA accumulation is sensitive to α-amanitin, starved cells were heat shocked at 40°C in the presence of 150, 300, or 600 ug/ml of α-amanitin. Tetrahymena, in a manner similar to other lower eukaryotes, requires a higher concentration of the drug to inhibit RNA polymerase II (Freiburg, 1981). The results of this experiment (Figure 12) show that at 600 ug/ml of α-amanitin, C5 RNA only accumulated to about 50% of non-treated cells. The level of hsp73, also on the same filter, accumulated to only 25% of non-treated cells. The level of 5s or G8 RNA, both transcribed by RNA polymerase III was only slightly affected (Kraus et al., 1987). These results indicate that either C5 RNA is not transcribed by RNA polymerase II or alternatively, C5 RNA transcription is less sensitive to the drug than is hsp73 transcription. No other example of this sort of differential sensitivity has been reported.
Sequence of the C5 cDNA

The pC5 series of cDNA clones isolated by Martindale and Bruns (1983) were cloned into pBS (shown in Figure 13) and sequenced using the Sanger dideoxy chain termination method (Sanger et al., 1977) (see Materials and Methods). Synthetic primers were available to the T7 and T3 promoter sequences allowing both strands of the DNA to be sequenced. The compiled sequence of the pC5 cDNAs are shown in Figure 14. The estimated size of the C5 transcript was 1450 bases, which is within 10 bases of the 1460 bases determined by sequencing. Reading frame analysis shows a potential open reading frame of 561 bases that could encode a peptide of 20.5 kD. This was determined by setting the codons TAA and TAG to be read as glutamine rather than stop codons. The ciliates have been shown to use these codons for glutamine in Tetrahymena histone H4 (Horowitz and Gorovsky, 1985), the actin gene from Tetrahymena, and the G and I surface antigens in Paramecium (Caron and Meyer, 1985, Preer et al., 1985, and Cupples and Pearlman, 1986). Recently, the tRNAs for the TAA and TAG codons have been isolated and sequenced for Tetrahymena (Hanyu et al., 1986). Of the published sequences of the known genes, the termination
The cDNA inserts from the pC5 series of clones that were originally isolated were all ligated into pBS for sequencing and in vitro transcription studies. The subclones of pC5.5 are shown as pBSLC5.7 and pBSLC5.3. The sizes of the inserts are shown next to the names.
<table>
<thead>
<tr>
<th>PBS-C5.1</th>
<th>685bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-C5.2</td>
<td>120bp</td>
</tr>
<tr>
<td>PBS-C5.6</td>
<td>465bp</td>
</tr>
<tr>
<td>PBS-LC5.3</td>
<td>360bp</td>
</tr>
<tr>
<td>PBS-LC5.7</td>
<td>740bp</td>
</tr>
<tr>
<td>PBS-C5.3</td>
<td>340bp</td>
</tr>
<tr>
<td>PBS-C5.4</td>
<td>350bp</td>
</tr>
<tr>
<td>PBS-C5.5</td>
<td>1104bp</td>
</tr>
<tr>
<td>C5 RNA</td>
<td>1460 bases</td>
</tr>
<tr>
<td></td>
<td>200 bp</td>
</tr>
</tbody>
</table>
codon used is TGA. However, the codon usage within this open reading frame does not conform to the codon usage found in either histone H3-H4, actin, or the ribosomal protein S25. In almost all organisms, codon usage is such that the first base of a triplet is a purine (R), the middle base either purine or pyrimidine (N), and the third base a pyrimidine (Y). The ratio of RNY to YNR codons is always greater than or equal to 2 (Shepherd, 1981). This relationship holds for *Tetrahymena* actin, histones, and ribosomal protein S25. The ratio of RNY to YNR codons in the open reading frame of C5 is less than 1. Also, there is a strong codon bias used by the ciliates. The best example of this is the codon for arginine which is, with one exception, AGA in all the ciliate genes sequenced so far, including those in *Paramecium*. Analysis of the codon usage for C5 showed that 5 of the 6 possible arginine codons are used. On the basis of these analyses, the likelihood that a peptide is encoded by the identified open reading frame seems doubtful. The structure of the C5 sequence is unusual in that it contains a large internal stretch of As at bases 700-740 and 1020-1101, interrupted 6 times by either A, T, C, or G. At the 3' end, there is a stretch of 50 As
The sequence of C5 was determined by sequencing the pC5 series of clones that were subcloned into pBS (see Materials and Methods). The open reading frame starts at base 1 and continues until base 561 where there is a TGA (*) (the open reading is underlined). The potential size of a peptide should be 20,570 daltons.
that is presumably a poly A tail and is interrupted six times with either or G or C. The average length of a poly A tail for *Tetrahymena* mRNAs has been estimated to be between 30 and 50 bases long (Calzone et al., 1983b).

**C5 is transcribed from a single gene**

The number of C5 RNA molecules accumulated in starved, heat shock, or stationary cells is certainly a significant amount. There are 200,000 molecules per cell of C5 RNA in long starved cells, and 140,000 molecules and 108,000 molecules per cell in 42°C heat shocked and stationary cells, respectively (see Table 2). We wanted to determine if all these transcripts come from one gene or from a multicopy family of genes. To determine the gene number, total genomic DNA was digested with a variety of restriction endonucleases. The digests were electrophoresed, transferred to a nylon membrane, and probed with the pC5.5 plasmid containing a 1.030 insert that represents the majority of the gene. The results of the autoradiogram of the Southern analysis are shown in Figure 15. The restriction enzymes Bam HI, Eco RI, Sal I, Eco RV, Kpn I, Sac I, and Pst I all produce a single fragment of DNA that hybridizes to the probe and do not cut within
Figure 15. C5 RNA is transcribed from a single copy gene

Total DNA was extracted from BIV cells and equal amounts were digested with the following restriction enzymes. Lane a-uncut, lane b-Eco RV, lane c-Bam H1, lane d-Alu I, lane e-Sal I, lane f-Hind III, lane g-Rsa I, lane h-Kpn I, lane i-Sac I, lane j-Pst I, lane k-Eco R1, lane l-Sau 3A, and lane m-Hinf I. Lambda DNA was digested with Eco R1 and Hind III as molecular weight markers. These DNA digests were electrophoresed on 0.8% agarose gels, transferred to membrane, and probed with $^{32}$P-nick translated pC5.5. The gel was run such that fragments larger than 500 base pairs would be retained.
the gene. Had there been more than one copy of the C5 gene, we would have expected multiple bands to appear on the autoradiogram from these digests. We cannot rule out that there are two copies of the C5 gene exactly duplicated with duplications of the restriction sites. We feel that this possibility is unlikely. The restriction enzymes Alu I, Hinf I, Rsa I, and Sau 3A all generate fragments that are smaller than the entire C5 RNA (1460). Analysis of the sequence shows multiple Alu I, Rsa I, and Sau 3A sites within the C5 cDNA, but only one Hinf I site. The gel was run so that fragments 500 base pairs and larger would be retained, therefore the small fragments generated from the above digests would not be seen or contain too little of the C5 gene to hybridize. The use of enzymes that cut within the gene also show that there is only one copy of the gene. Multiple copies of C5 would have generated many more fragments when *Tetrahymena* DNA digested with enzymes that cut within the C5 gene. Therefore, one gene is responsible for the large number of transcripts of C5 RNA molecules (see Discussion).
Conservation of the C5 gene in other species and stains of Tetrahymena

We obtained 5 species of Tetrahymena (T. elliottii, canadensis, tropicalis, and australis, T. pyriformis) to determine the extent of C5 gene been conservation. We also examined three T. thermophila strains that lack a micronucleus on the basis that if the C5 gene product is involved in the process of conjugation, then it might be present in the micronucleate (fertile) species and absent in the amicronucleate (sterile) species and stains. T. pyriformis, an amicronucleate sterile species, was included in this study. We also digested DNA from the star strains, A* and B*, and an amicronucleate mutant of T. thermophila (Kaney and Speare, 1983 and Orias and Bruns, 1974) that are still able to form pairs and receive a micronucleus during conjugation. Total genomic DNA was isolated from each cell type, digested with Eco R1, transferred to nylon membranes, and hybridized with a subclone of pC5.5, termed pBSLC5.3, that contains 360 base pairs of the 5' end of the gene (see Figure 13). We have found that the long poly A tracts in pC5.5 contribute to a background of non-specific hybridization to A-rich regions in the genome (S. Libby, Department of Zoology,
Figure 16. Conservation of the C5 gene in other species and strains of Tetrahymena

(A) Conservation of the C5 gene in other species of Tetrahymena. Total DNA was extracted from 4 different species of Tetrahymena, 2 amicronucleate strains of T. thermophila (A*, B*), BII (micronucleate strain used in this paper), and T. pyriformis. The DNA was digested with Eco RI, separated on 0.8% agarose gels, transferred to nylon membrane, and probed with P32-nick translated pBS-LC5.3. The lanes on the resulting autoradiogram are as follows. (mic=micronucleate fertile strain and amic=amicronucleate sterile strain). Lane a-T. australis mic, lane b-T. australis amic, lane c-T. canadensis mic, lane d- T. canadensis amic, lane e- T. tropicalis amic, lane f-T. tropicalis mic, lane g-T. elliotti mic, lane h-T. elliotti amic, lane i-A*, lane j-B*, lane k-BII, and T. pyriformis. Lambda Hind III was used as a size marker and is shown on the far right. (B) Amicronucleate strains of thermophila accumulate C5 RNA when starved. RNA was extracted from growing and starved A*, Ag, and BIV cells (micronucleate), electrophoresed, transferred to nylon a membrane, and probed with pC5.5. Lanes 1, 3, and 5 is RNA extracted from growing A*, BIV, and Ag cells. Lanes 2, 4, and 6 is RNA from long starved cells. A* and Ag cells accumulate a RNA that hybridizes to pC5.5 and is the same size as the C5 RNA in BIV cells.
Iowa State University, Ames, IA unpublished observations). When BII, the strain used in all experiments described in this paper, is digested with Eco R1 and hybridized with pBSLC5.3, a single band at 7.6 kb hybridizes (Figure 16). The same size fragment similarly hybridizes to B* and A* DNA, except that an intensely hybridizing fragment of 4.2 kb also is apparent in A*, but not in B*. These same size fragments also hybridizes to DNA from both T. canadensis micronucleate and amicronucleate strains. There are at least 6 fragments of DNA that hybridize to T. canadensis, DNA suggesting that the C5 gene in this species is present in multiple copies. The C5 specific probe did not hybridize to any DNA in T. tropicalis, australis, ellioti, or pyriformis. This filter was re-hybridized and washed at reduced stringency, and still no hybridization to these mentioned strains was detected (data not shown). Since the amicronucleate strains A* and Ag (Kaney and Speare, 1983) are still able to form pairs during conjugation, we determined whether these strains accumulate C5 RNA when starved. The Northern analysis of RNA from growing and starved A*, Ag, and BIV is shown in Figure 15b. When starved, these cells accumulate an RNA that hybridizes to pC5.5
and has the same size as C5 RNA from BIV cells. However, when growing, these cells do not accumulate C5 RNA. Whether or not both amicronucleate and micronucleate strains of *T. canadensis* are able to accumulate a C5-like RNA when starved remains to be determined, but on the basis of the strength of hybridizations to the DNA, we predict that the cells probably would. To rule out the possibility that the C5 gene product is not involved in some aspect of conjugation will require finding a sterile strain of *T. thermophila* and probing its DNA with pC5 and with the two conjugation specific clones pC1 and pC2.
DISCUSSION

C5 RNA accumulates only when cells are not dividing

We find that C5 RNA becomes a predominant poly A+ RNA during conditions that cause cell division to be arrested in Tetrahymena thermophila. This RNA is not present in detectable amounts in growing cells. As long as cells are prevented from dividing either by prolonged starvation or heat shock, C5 RNA levels remain constant; however, when cells are allowed to resume dividing the C5 RNA level drops rapidly.

Starvation is a physiological condition that changes cell metabolism drastically causing 96% of the poly A+ RNA to be unloaded from polysomes. In addition, ribosome content drops by 20% with no net accumulation, and cell division is arrested (Sutton and Hallberg, 1979 and Calzone et al., 1983a). Yet, starvation induces a rapid accumulation and maintenance of high steady state levels of C5 RNA such that it comprises 7.6% of the poly A+ RNA (Figure 2 and Table 1). The concentration of C5 RNA is starved cells is 150 time greater than histone H4 RNA, the only other mRNA for which measurements of this kind have been done.
We have calculated the minimum number of transcripts per minute that would be needed for C5 RNA to accumulate to high levels in starved cells. Since there is one gene per haploid genome and the Tetrahymena genome is 50n, there are a total of 50 genes. Therefore, the ratio of C5 RNA molecules to genes is 4000 to 1 and C5 RNA is transcribed minimally at 67 molecules/gene/minute. By comparison, ribosomal RNA genes are transcribed at 167 molecules/gene/minute (2 x 10^6 ribosomes per cell/20,000 gene/60 minutes) and the histone H4 genes are transcribed at 0.3 molecules/gene/minute (1600 molecules/100 genes/60 minutes) (Bannon et al., 1983). These estimates for C5 and histone H4 must be minimum estimates since these RNAs are not as stable as ribosomal RNA. Even so, the transcription rate of C5 RNA is significant, being only about 2.5 time less than that of ribosomal RNA transcription. Whether any other RNA is induced to accumulate to the extent of C5 RNA in starved cells or transcribed at this rate is unknown to the lack of such analysis.

As long as cells are starved (24 hours in this study), the steady state level of C5 RNA remains constant. When starved cells are stimulated to resume
division by refeeding, the level of C5 RNA drops very rapidly with in the first 1 hour, and by 2 hours, the level has fallen to less than 1% of the amount in long starved cells. This rapid turnover of C5 RNA takes place in the absence of cell division. Starvation and stationary growth are both cases when overall cellular metabolism is depressed. In both cases, there are fewer messages loaded onto polysomes, ribosome content drops, and overall protein and RNA synthesis is depressed. Although we do not wish to imply that there is a cause and effect relationship between the drop in ribosome content and the accumulation of C5 RNA, there does exist this correlation. Clearly, there are complex metabolic changes that take place during these conditions that are responsible for the regulation of metabolism of C5 RNA.

The time required for cells to resume division and the level of C5 RNA to fall in 39°C and 40°C heat shocked cells correlates with the changes in the pattern of expression of the heat shock genes. When cells are heat shocked at 39°C and 40°C, cell division is temporally interrupted and C5 RNA accumulates rapidly. The same holds true when cells are heat shocked at 41°C or 42°C, with respect to the heat shock
response, but cells are not able to resume division and the level of C5 RNA remains constant. When cells heat shocked at 42° or 43°C are returned to 30°C, cell division is able to begin, and the level of C5 RNA drops rapidly during recovery prior to the beginning of cell division. During heat shock, the regulation of C5 RNA metabolism is temperature dependent as is the effect on cell division. Even though at the gross level, the heat shock response appears to be similar, there must be some component(s) that regulate cell division that are temperature sensitive.

C5 RNA accumulation and maintenance requires ongoing protein synthesis, but not continued DNA replication.

A metabolic change that occurs during heat shock, starvation, and in stationary cells is the interruption of DNA replication. Our data suggest that the primary signal for C5 RNA induction is independent of continued DNA replication. We could not cause the induction of C5 RNA in growing cells when DNA replication was inhibited with aphidicolin. Also, 40°C heat shocked cells, treated with aphidicolin metabolized C5 RNA in the same manner as did non-treated, heat shocked cells. Similarly, refed starved cells that were prevented from
dividing by blocking DNA replication regulated C5 RNA levels as in non-treated cells. In stationary cells, the rate of division slows but does not stop completely. As cell densities reach greater than 300,000 cell/ml, C5 RNA begins to be accumulated (Figure 7). These data suggest that a component of the signal that causes C5 RNA to be induced may be a change in the rate of cell division. Whatever the metabolic signal is that causes the transcription of the C5 gene, it is not coupled to or dependent on ongoing DNA replication. In fact, we have not been able to cause the accumulation of C5 RNA in growing cells except by starvation, heat shock, or letting the cells become stationary.

We have shown C5 RNA accumulation and maintenance has an absolute requirement for protein synthesis. That C5 RNA levels are sensitive to the protein synthesis inhibitor cycloheximide is unique as compared to other mRNAs. Cycloheximide has been shown to stabilize histone mRNA (Slve et al., 1984 and Stimac et al., 1984), heat shock mRNAs (DiDomenico et al., 1982), and even cause c-myc RNA to become superinduced (Linial et al., 1985). Cycloheximide sensitivity appears to be specific to C5 RNA as the level of another RNA, C6
(surface antigen) remains constant during the same starvation conditions with cycloheximide. Similarly, the level of HSP 70 mRNA during heat shock at 41°C is not affected by cycloheximide and, if anything, is stabilized during heat shock. Conversely, while C5 accumulation, maintenance, and recovery, shows the same sensitivity as in starvation (data not shown).

As we can only measure net accumulation using Northern and slot blot analysis, C5 RNA could be transcribed in the presence of aphidicolin or cycloheximide, but is turned over so rapidly that it never accumulates to a detectable level. Nuclear run off experiments with aphidicolin and cycloheximide treated cells should tell us whether the manifestation of the drug is at the level of transcription or regulated by post-transcriptional mechanisms. Also, the level of regulation of C5 RNA during refeeding, heat shock, and starvation could be determined using this technique. This type of analysis has been used to determine that the level of control of histone mRNA in cycloheximide treated cells is at the level of transcription (Slve et al., 1984).
Evidence that C5 RNA is not a messenger RNA

Some of the properties of C5 RNA suggest that it could be a mRNA. Its retention on an oligo d(T) column and subsequently being cloned as a cDNA are two classical definitions of poly A+, mRNA. However, more compelling evidence that C5 RNA is not a mRNA is as follows. First, in the presence of α-amanitin, a RNA polymerase II inhibitor, the level of C5 RNA in starved, heat shocked cells was reduced 30-50%, while the level of a known mRNA (hsp73) was reduced 85-90% of the level of non-treated cells. Second, the association of C5 RNA with polysomes is unusual. The amount of C5 RNA loaded (or associated) with polysomes never exceeds 30% of the C5 RNA in long starved cells. In the same preparations of polysomes, the majority of RNA encoding a putative surface antigen, pC6, is loaded onto heavy polysomes in early and long starved cells. Also, experiments with conjugating cells show that the two conjugation specific RNAs sediment in the heavy polysome region of the gradient. Third, since there are 200,000 molecules of C5 RNA in long starved cells and at least 25% of the molecules are loaded onto polysomes, 50,000 molecules of C5 RNA are in the polysome region. There are only 250,000 loaded
messages in long starved cells and C5 would comprise one-fourth of these. If this is really true, then we would expect a protein to be accumulated in a proportional amount. We have not detected any protein in starved cells with this characteristic.

Analysis of the codon usage in the open reading frame of C5 cDNA does not conform to either that used by known ciliate genes, or follow the general rule for most genes sequenced from a variety of organisms. On the basis of codon usage for arginine, the ciliates use AGA exclusively and not the other 5 potential codons. In the open reading frame of C5, 5 or 6 arginine codons could be used. Also, the total number of codons used out of 64 is reduced in ciliate genes. The range of the number of codons used from histone H4 to actin is 29-43. The potential number of codons used in C5 is 54, which is more like codon usage seen in mammalian genes. Taken together, the unusual characteristics of the potential reading frame and those already mentioned exclude the possibility that C5 codes for a peptide. To determine if C5 RNA could be translated, we could use hybrid selected C5 RNA in an in vitro translation assay.
We cannot rule out that the large amount of C5 RNA in total RNA isolates nor that which sediments with the polysomes is due to contamination of heteronuclear RNA. To prepare polysomes, the cells are lysed by mechanical shearing with no detergent present. The intact nuclei are visible in the lysate under the microscope. Also, most of the nuclei should be pelleted in the first 10,000 rpm spin to remove cell debris so that contamination with nuclear contents should be minimal.

Finally, there are few if any examples in the literature describing the biological characteristics of any other RNA like C5 RNA from *Tetrahymena*. The amount of C5 RNA accumulated in non-dividing cells suggests that it is playing some role, at least in *Tetrahymena thermophila* and maybe in *Tetrahymena canadensis*. Even though the other species of *Tetrahymena* are closely related based on their 17s rRNA sequence, the gene for C5 appears not to be conserved (Elliott, 1973; D. Nanney, manuscript submitted University of Ill, Urbana, Ill.). Exactly what this role is remains to be determined.
LITERATURE CITED


ACKNOWLEDGMENTS

I would like to thank Dr. Richard Hallberg for his patience and guidance throughout this study. I would also like to thank Drs. Clark Ford, John Mayfield, Bob Andrews, and Duane Enger for their helpful suggestions. I also wish to thank Tom McMullin, Kevin Kraus, and Don Reading for their friendship, moral support, and badgering. Finally, I thank my wife, Stephanie, for her never-ending patience and constant encouragement.