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Starved *Tetrahymena thermophila* Cells That Are Unable to Mount an Effective Heat Shock Response Selectively Degrade Their rRNA

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***Tetrahymena thermophila* cells that had been shifted from log growth to a non-nutrient medium (60 mM Tris) were unable, during the first few hours of starvation, to mount a successful heat shock response and were killed by what should normally have been a nonlethal heat shock. An examination of the protein synthetic response of these short-starved cells during heat shock revealed that whereas they were able to initiate the synthesis of heat shock proteins, it was at a much reduced rate relative to controls and they quickly lost all capacity to synthesize any proteins. Certain pretreatments of cells, including a prior heat shock, abolished the heat shock inviability of these starved cells. Also, if cells were transferred to 10 mM Tris rather than 60 mM Tris, they were not killed by the same heat treatment. We found no abnormalities in either heat shock or non-heat shock mRNA metabolism in starved cells unable to survive a sublethal heat shock when compared with the response of those cells which can survive such a treatment. However, selective rRNA degradation occurred in the nonsurviving cells during the heat shock and this presumably accounted for their inviability. A prior heat shock administered to growing cells not only immunized them against the lethality of a heat shock while starved, but also prevented rRNA degradation from occurring.**

Similar to other eucaryotic organisms, the ciliated protozoan *Tetrahymena thermophila* responds to certain stresses (e.g., elevated temperature, deciliation, or release from anoxia) by inducing the synthesis of a group of about 12 to 15 proteins (heat shock proteins [hsp's]) while simultaneously suppressing the synthesis of "normal" proteins (6, 7, 11, 30). Although it is not clear what specific functions the heat shock response and these induced "stress proteins" carry out, the evolutionary conservation of their synthesis in response to a variety of environmental and developmental conditions indicates that these proteins are, at times, crucial to cell survival (reviewed in reference 25).

Although usually expressed as a group, there is now good evidence that the synthesis of these proteins can be independently regulated (3, 17, 31). With that in mind, we were interested in knowing whether one particular stress, heat, always elicited from cells the same protein-synthetic response (either qualitatively or quantitatively) no matter what their physiological state might be. In the course of this study we found an unexpected situation in which a normally nonlethal temperature, but one which was sufficient to induce hsp synthesis, would kill these cells. Characterization of this condition revealed that the inability to survive was not due primarily to an inability to synthesize stress (or heat shock) proteins but apparently to the loss of ability to synthesize adequate amounts of these proteins. We investigated two possible causes for this: inadequate production of mRNAs for hsp's and inactivation of the protein-synthetic machinery. No aberrant mRNA metabolism, for either hsp's or non-hsp's, was found. However, the sensitive cells showed a selective degradation of rRNA as a result of the heat treatment, presumably accounting for the loss of protein-synthetic activity.

MATERIALS AND METHODS

Culture conditions. In all experiments we used a single strain of *T. thermophila*, CU355 (IV). We routinely grew cells at 30°C on a gyratory shaker in 1% Difco Proteose Peptone–0.003% Sequestrene (Ciba-Geigy). We considered cells to be in early log cell growth only if they were present at, at most, 100,000/ml and doubling every 2.75 h or less. Cells which had reached a density of at least 800,000/ml were considered to have entered "plateau," although they would continue to increase in cell number after this, albeit at a much reduced rate (doubling time, >20 h). The two standard starvation media we used were 0.06 M Tris-chloride pH 7.5 ("60 mM Tris"), and 0.01 M Tris-chloride, pH 7.5 ("10 mM Tris"). Both have a lower osmolarity than 1% Difco Proteose Peptone. Other starvation media used differed in the following ways: (i) they contained different concentrations of Tris but in all cases were adjusted to pH 7.5; (ii) they contained NaCl at various concentrations in addition to Tris but were always adjusted to a pH of 7.5; (iii) instead of Tris as a buffer they contained 0.001 M PO_4 (H_2NaPO_4-HNa_2PO_4) and were also adjusted to a pH of 7.5. Cells were transferred from growth medium into starvation media and vice versa as previously described (14). Heat shocks were administered to cells by transferring culture flasks from a 30°C incubator to a 38 to 41°C shaking water bath. We used volumes of cell suspensions such that the temperature shift was complete within 3 min of the transfer.

When treating cells with cycloheximide, we used a concentration of 5 μg/ml to inhibit protein synthesis. This concentration of drug brings about a >97% reduction in rate of protein synthesis within 2 min at both 30 and 41°C.

Cell viability measurements. The effect of heat shock on the viability of cells in starvation media was determined in the following way. Cell cultures (usually 10 to 15 ml) were incubated at 38 to 41°C for 90 min. They were then transferred to a 30°C incubator. Small samples of cells were examined under a dissecting microscope at intervals thereafter to determine the swimming behavior of the cells. The actual number of viable (swimming) cells was determined by diluting the cell suspensions to a point where they could be examined in a hemacytometer and an accurate count of swimming cells could be made. Corrections were made for

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the dilution, and the swimming cells per milliliter of the original culture was calculated. We found that the number of swimming cells often increased during the recovery at 30°C, but this only occurred during the first hour or two. Consequently, we made all of our measurements at 4 h or more post-recovery. As the cells were in starvation media, no cell division occurred and the number of viable cells at 4 h post-recovery was the same as that determined at 12 to 18 h post-recovery. Therefore, cell viability counts were often made after an overnight recovery.

Labeling of cell proteins. To label the proteins of cells in either growth media or starvation media, we used [^{3}H]lysine ([4,5-^{3}H]lysine; 80 Ci/mmol; Amersham Corp.) as a precursor. This amino acid was chosen because its concentration in proteose peptone is extremely low. For fluorographic analysis of cell protein, we typically labeled 10^6 cells in 3 to 6 ml of medium containing 10 to 20 μCi of [^{3}H]lysine per ml. Labeling periods ranged from 10 to 30 min. At the end of the labeling the cells were collected and processed as described below.

To determine the rate of incorporation of amino acids at times during heat shock and starvation, we pulse-labeled cells for 10 min with 20 μCi of [^{3}H]lysine per ml at various intervals. At the end of a pulse, three 100-μl samples were removed and each was mixed with 100 μl of 0.05 M NaOH to lyse the cells. A 3-ml portion of cold 10% trichloroacetic acid was then added to precipitate the proteins, which were subsequently collected and washed on glass fiber filters. Filters were counted in a liquid scintillation counter and the average value for each time point was determined. Zero-time background incorporation was subtracted from each time point.

Protein electrophoresis. Total cell proteins were prepared for electrophoretic analysis essentially according to the method of Guttman et al. (11). A volume of a particular cell suspension containing approximately 10^6 cells was centrifuged to pellet the cells, which were then suspended in 1 ml of 10 mM Tris, pH 7.5. The cells were repelleted, the washing solution was aspirated off, and 100 μl of lysis buffer (0.05 M Tris, pH 6.8, 1.5% sodium dodecyl sulfate [SDS], 7.5% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin) was added. After mechanical disruption of the cell pellet by vortexing, the lysate was heated to 100°C for 3 min. If not immediately used for electrophoresis, samples were stored at −70°C.

One-dimensional gel electrophoresis was performed according to Guttman et al. (11). SDS-containing 15 to 20% polyacrylamide slab gels were used in all cases. After the stacking gel had polymerized, the gel was cooled to 4°C for 1 h and then prerun at 10 mA per gel for an additional hour. Samples of the cell lysates, 10 μl per lane, were electrophoresed at 4°C for 8 h at 20 V/cm. Subsequently, gels were stained with 0.05% Coomassie brilliant blue R in 50% methanol–10% acetic acid, destained in 10% methanol–10% acetic acid, and fluorographed (25).

Plasmids used. (i) Heat shock genes. The two clones used as probes for heat shock mRNAs for hsp73 and hsp80 were subcloned into pBR322 from larger genomic clones isolated from a lambda phage library prepared from *T. thermophila*. The hsp73 clone is a 1.0-kilobase insert and has been identified by DNA sequence analysis (R. C. Findly, manuscript in preparation). Preliminary analysis of the second clone indicates that it codes for hsp80 (Findly, unpublished data).

(ii) Non-heat shock cDNAs. Five pBR322 plasmids containing cDNA inserts were used. Their preparation from *T. thermophila* mRNAs and characterization have been published (19). Each plasmid hybridizes to a unique-sized RNA. The plasmid designations and the sizes of the mRNAs which they code for are as follows: pC3-2, 430 base pairs (bp); pC4-1, 560 bp; pC5-5, 1,450 bp; pC6-1, 1,470 bp; pC8-1, 4,200 bp. A pBR325 plasmid (pDP6) containing a chromosomal 5S rRNA gene was obtained from D. Pederson (University of Rochester). It has a 3.8-kilobase insert which contains a single copy of a complete 5S rRNA gene. When hybridized to total cellular RNA it reacted only with an RNA species of approximately 100 to 150 bp.

(iii) Plasmid isolation and labeling. Plasmids were isolated from SDS-lysed bacteria according to the procedure of Godsen and Vapnek (9). They were subsequently purified on CsCl-ethidium bromide density gradients. Purified plasmid DNAs were nick translated, using a kit from Amersham Corp., to specific activities of 6.0×10^7 to 7.5×10^7 cpm/μg of DNA.

RNA isolation. (i) Total RNA. All solutions coming into contact with RNA were autoclaved, and all glassware was heat treated at 165°C for at least 4 h. Cells (10^6 to 10^7) were collected by centrifugation and lysed with 2 to 5 ml of RNA lysis buffer (0.1 M NaCl, 10 mM EDTA, 10 mM Tris-chloride [pH 7.4], 1% SDS, 0.5 mg of heparin per ml). After 5 to 10 min in lysis buffer at room temperature, protein was removed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction followed by two chloroform-isoamyl alcohol (24:1) extractions. The RNA was precipitated at −20°C by adding 0.1 volume of 3 M sodium acetate (pH 7) and 2.5 volumes of 95% ethanol. After centrifugation at 12,000 × *g* for 15 min, the pellet was washed with ice-cold 70% ethanol, dried, and dissolved in distilled water or TE buffer (10 mM Tris-chloride [pH 8], 1 mM EDTA).

(ii) rRNA. High-salt (0.6 M KCl), washed ribosomes were prepared from log-phase cells as described by Hallberg et al. (14). RNA was extracted from pelleted ribosomes as described for whole cells.

RNA electrophoresis. (i) For Northern transfers. RNA was denatured with deionized glyoxal and dimethyl sulfoxide (21) and run as described on 1.5% agarose gels in 10 mM NaH_2PO_4 (pH 7.0) at 3 to 4 V/cm for 3 to 4 h. Buffer was intermittently circulated between the cathodic and anodic chambers to prevent a pH gradient being established. The resulting gels were stained with 1 μg of ethidium bromide per ml of distilled water and were then photographed while illuminated with near-UV irradiation.

(ii) For visualization of RNA. RNA in TE buffer was heated to 60°C for 2 to 3 min. Such treatment disrupts the secondary structure of the rRNA, causing release of the 5.8S rRNA and breakdown of the 26S rRNA into two fragments. The RNAs were run on 2.2% agarose gels in 0.089 M Tris-borate (pH 8.0)–0.089 M boric acid–0.002 M EDTA at 3 to 4 V/cm for 5 to 6 h. They were stained and photographed as described above.

Gel-to-filter transfers and filter hybridizations. Stained gels were washed in distilled water for about 1 h to remove most of the ethidium bromide. The glyoxylated RNA was transferred to either a presoaked (in 2× SSC [SSC = 0.15 M NaCl plus, 0.015 M sodium citrate]) Genatran 45 (D and L Filter Corp.) or Genescreen (New England Nuclear Corp.) filter according to Thomas (29). After transfer, filters were baked in a vacuum oven for 2 h at 80°C. The filters were prehybridized for 18 to 20 h at 42°C in a solution containing 50% formamide (Sigma Chemical Co.), 5× SSC, 1× Denhardt solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 25 μg of yeast-soluble RNA (type III;

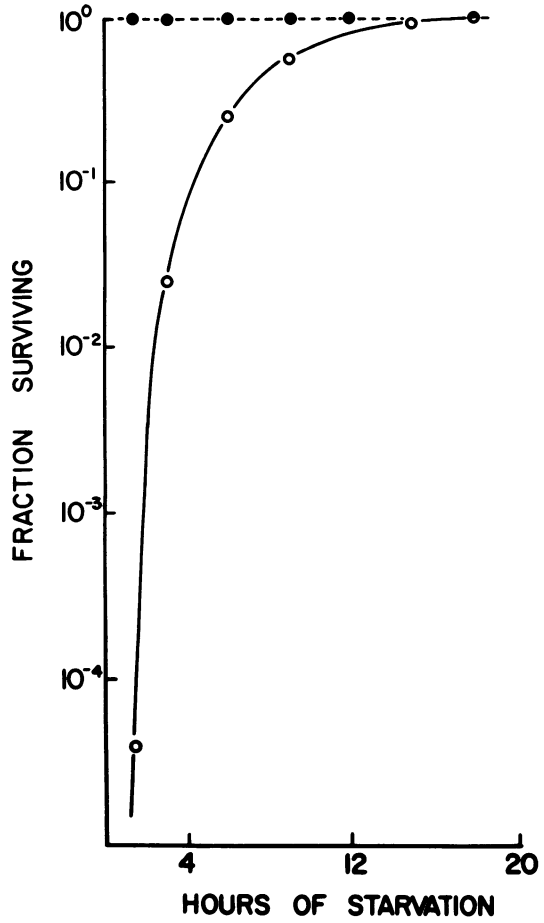


FIG. 1. Change in ability of cells in starvation medium to survive a 90-min heat shock. Cells in early log growth were washed into starvation media at 30°C. At various times thereafter, samples of cells were removed and transferred to 41°C for 90 min. The cultures were transferred back to 30°C, and the fraction of surviving cells was measured 2 to 4 h later. Symbols: (○) cells starved in 60 mM Tris; (●) cells starved in 10 mM Tris.

Sigma) per ml, 250 μg of denatured herring sperm DNA (Sigma) per ml, and 0.2% SDS. Labeled probes (ca. 10^6 cpm per filter) and dextran sulfate (10% final concentration; Sigma) were added directly to the prehybridization mixture, and hybridization was carried out at 42°C for 12 to 20 h. The filters were washed and exposed to X-ray films (Kodak XS-5 or XAR-5) as described by Thomas (29). When filters were later hybridized to another probe (usually the one containing the 5S rRNA gene), they were taken through the entire procedure a second time.

Quantitation of autoradiograms. Appropriately exposed X-ray films (those in which the density of exposure was <2.5 absorbance units) were scanned on an LKB Ultrascan densitometer. Peak areas were determined by tracing the scans, cutting out the peaks, and weighing them. All values for a given RNA species on a particular autoradiogram were normalized to a presumed constant amount of 5S rRNA measured on an autoradiogram produced from that same filter (31).

RESULTS

In the course of comparing the response of *T. thermophila* cells that were heat shocked while exposed to a variety of different physiological conditions, we came upon an exceptional case. Among others, these conditions involved transferring *T. thermophila* from growth medium to either of two non-nutrient media: (i) 10 mM Tris, a treatment which induces conjugation; or (ii) 60 mM Tris, which simply starves the cells. *T. thermophila* cells remain viable for at least 5 days under either of these starvation conditions. When early log-phase cells were washed from growth medium, suspended in 60 mM Tris, allowed to starve for up to 1 h, and then heated to a temperature which should have induced a nonlethal heat shock response (38 to 41°C), they rounded up, began swimming slowly, and died (<0.01% survival) within 60 to 90 min. However, cells starved in 60 mM Tris eventually acquired the ability to survive a heat shock in this medium (Fig. 1). It was not the starvation, per se, which prevented cells from surviving a heat shock, as log cells washed into 10 to 40 mM Tris survived a 90-min heat shock shortly after resuspension in starvation medium (Table 1; Fig. 1). These cells also showed no change in morphology or swimming behavior as a result of heat shock. In 50 mM Tris, 2% of the cells survived a 90-min heat shock, but at any higher Tris concentration in which cells were viable at 30°C a heat shock proved to be lethal. This was not an effect of Tris itself since keeping the concentration of Tris at 10 mM and increasing the concentration of NaCl in the starvation medium, or using a starvation medium without Tris, demonstrated that the lethality of a heat shock of <41°C is associated with the ionicity of the medium (Table 1). However, cells in 1% Difco Proteose Peptone supplemented with 70 mM Tris or NaCl were not killed by a 41°C heat shock.

The history of the cells just before suspension in starva-

TABLE 1. Heat shock viability in different media

Media	Survivors (%)[a]
Starvation[b]	
Tris alone	
10, 20, 30, or 40 mM Tris	100
50 mM Tris	2
60 or 70 mM Tris	<0.01
10 mM Tris plus NaCl	
10, 20, or 30 mM NaCl	100
40 mM NaCl	5
50 or 60 mM NaCl	<0.01
1 mM PO_4 plus NaCl	
0 or 10 mM NaCl	100
30 mM NaCl	81
50 or 70 mM NaCl	<0.01
Nonstarvation[c]	
1% Proteose peptone plus 70 mM Tris	100
1% Proteose peptone plus 70 mM NaCl	100

[a] Survivors are defined as cells still alive and swimming in 60 mM Tris 12 h after having been removed from the heat shock conditions. The percent survivors was determined by comparing the number of swimming cells surviving a heat shock in a particular medium with those treated in a similar manner except for the heat shock.

[b] In all experiments utilizing starvation media (see text for details regarding the different media), cells at 30°C in early log growth (<50,000/ml) were pelleted from growth media, suspended in a particular starvation medium, washed once by centrifugation, and then resuspended in the same starvation medium. The cells were then transferred to 41°C for 90 min.

[c] To cells in early log growth in 1% peptone was added a 1/20 volume of a 20× salt solution, and cells were then heat shocked for 90 min at 41°C. After the heat shock, cells were collectd by centrifugation and washed into 60 mM Tris at 30°C to prevent any further cell division from occurring.

TABLE 2. Acquisition and loss of ability to survive a heat shock in starvation medium

Condition	Time (min) at 41°C in peptone before a 90-min heat shock in 60 mM Tris	Time (min) at 30°C in peptone after a 60-min heat shock at 41°C	Survivors (%)[a]
Acquisition of heat shock viability[b]	0		<0.01
	10		0.2
	20		1.8
	30		9
	45		46
	60		98
	90		96
Loss of heat shock protection[c]		0	100
		30	65
		60	29
		90	1.2
		120	0.1
		150	<0.01

[a] After a 90-min heat shock at 41°C, cells were transferred to 30°C and allowed to recover for at least 4 h (as the cells are in starvation media, no cell division will occur). The number of swimming cells was determined and compared with the number in a culture which had been starved but not heat shocked. This ratio (as a percentage) determined the fraction of cells surviving the heat shock.

[b] Cells in early log growth (<50,000/ml) at 30°C were shifted to 41°C for various lengths of time before being washed into 60 mM Tris and returned to 41°C for 90 min.

[c] Cells in early log growth at 30°C were heat shocked for 60 min at 41°C. They were then transferred back to 30°C, and at various times thereafter cells were washed into 60 mM Tris and heat shocked at 41°C for 90 min.

tion medium was important in determining whether they could survive a heat shock in 60 mM Tris. In contrast to early log-phase cells, cells in a mid- to late-log stage of growth (ca. 600,000/ml), when transferred to 60 mM Tris, survived at a level of 25 to 30% after a 90-min heat shock, whereas cells in early plateau (>800,000/ml) displayed a 100% survival when subjected to the same treatment. In both of these cases, the cells rounded up and reduced their rate of swimming during the first hour of heat shock but then returned to a more fusiform shape and normal swimming behavior within 90 min at the elevated temperature. Cells starved in 60 mM Tris for 18 h, refed with concentrated growth medium, and then returned to 60 mM Tris and heat shocked showed a 100% survival if they had been refed for 1 h or less. However, by 2 h of having been refed, <0.01% of the cells survived a 90-min heat shock of >38°C. This abrupt loss of resistance to heat shock killing occurred in a nondividing population of cells (12).

There was one situation which allowed growing cells to be briefly starved in 60 mM Tris and survive a heat shock: if they had been previously heat shocked while in growth media. This thermoprotection (probably not analagous to acquired thermotolerance [18, 20, 22]) required a full hour of prior heat shock to manifest itself fully (Table 2). Moreover, this protection was transient. As cells were allowed to recover at 30°C in growth medium before being starved and again heat shocked, they gradually lost their thermoprotection (Table 2). Unlike growing cells transferred to 10 mM Tris and heat shocked, these survivors underwent the transient morphological and swimming change described before. As before, cells that survived the heat shock began to recover their normal shape and swimming patterns after 1 h, even at the elevated temperature.

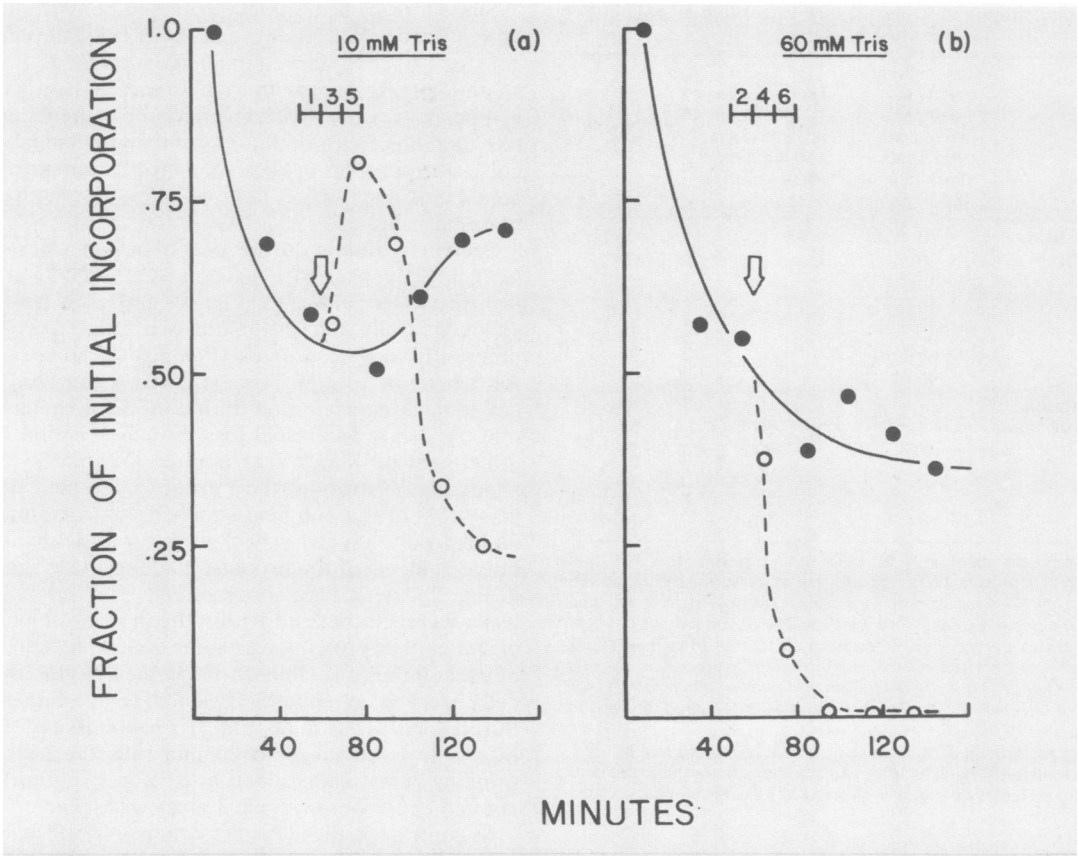
Since a prior heat shock protected cells from heat shock killing in 60 mM Tris, we examined cells starved in 10 mM Tris for 1 h, cells starved in 60 mM Tris for 18 h, and cells in a late-log stage of growth for evidence of heat shock protein synthesis as a possible explanation for their thermoprotective qualities. With both long-term and pulse-labeling protocols, we found no evidence on two-dimensional polyacrylamide gels that such conditions elicit the synthesis of hsp's (data not shown).

Protein synthesis during heat shock of starved cells. We next directly examined protein synthesis in growing cells transferred to either 60 or 10 mM Tris and then heat shocked. Cells starved in the two media responded quite differently to a heat shock (Fig. 2a). Whereas cells starved for 1 hour in 10 mM Tris exhibited a transient increase in amino acid incorporation during the heat shock, those in 60 mM Tris showed a rapid loss in incorporation immediately after the heat shock was applied. Within 35 min of heat shock administration, the rate of amino acid incorporation was <1% of the non-heat-shocked controls, and by 55 min no incorporation above background was detectable. A qualitative analysis of the proteins made at these times is shown in Fig. 2b. After heat shock of cells starved in 10 mM Tris, hsp's were synthesized within the first 10 min and synthesis of most other proteins was somewhat reduced. This pattern of hsp synthesis is similar to that seen in growing cells or in cells starved in 60 mM Tris for 18 h (data not shown). Surprisingly, cells in 60 mM Tris also initiated the synthesis of hsp's. Even at 10 to 20 min into the heat shock, the proteins being made, albeit at <5% the rate in 10 mM Tris-starved cells, were a typical array of hsp's.

In contrast to these results, when growing cells which had previously been heat shocked for 1 h were washed into 60 mM Tris and heat shocked again, they showed an apparently normal hsp synthesis response (data not shown). Thus, cells which are unable to survive a heat shock in 60 mM Tris do not die because they cannot switch to synthesizing hsp's but presumably because they cannot synthesize a sufficient amount of these proteins. That it is the case that short-starved cells will die during a heat shock if they do not synthesize hsp's is shown by the fact that cells starved for 1 h in 10 mM Tris, when heat shocked at 41°C in the presence of cycloheximide (5 μg/ml), were killed. Interestingly, it took a while longer (120 to 150 min) for these cells to die, and they did not show the morphological change during this time which is so apparent in heat-shocked, 60 mM Tris-starved cells.

mRNA metabolism during heat shock. The inability to mount a quantitatively effective heat shock response was presumably due either to the underaccumulation of functional heat shock messages or to a more general breakdown in protein synthetic capacity. Therefore, we first examined the metabolism of heat shock and non-heat shock mRNAs during a normal heat shock response. These data would serve as a basis for a subsequent comparison to the mRNA metabolism of heat-shocked, short-starved cells. Figure 3 shows the changes in relative concentrations of messages for hsp73 and hsp80 and five non-heat shock mRNAs during the course of a continuous heat shock. The changes in levels of the hsp mRNAs during a recovery are also shown. Both hsp messages were detected at low levels in log cells, and their levels increased considerably upon heat shock. Their concentrations decreased rapidly upon return to 30°C.

The levels of non-hsp mRNAs displayed a variety of changes in response to the heat shock (Fig. 3). These changes, for both heat shock and non-heat shock mRNAs,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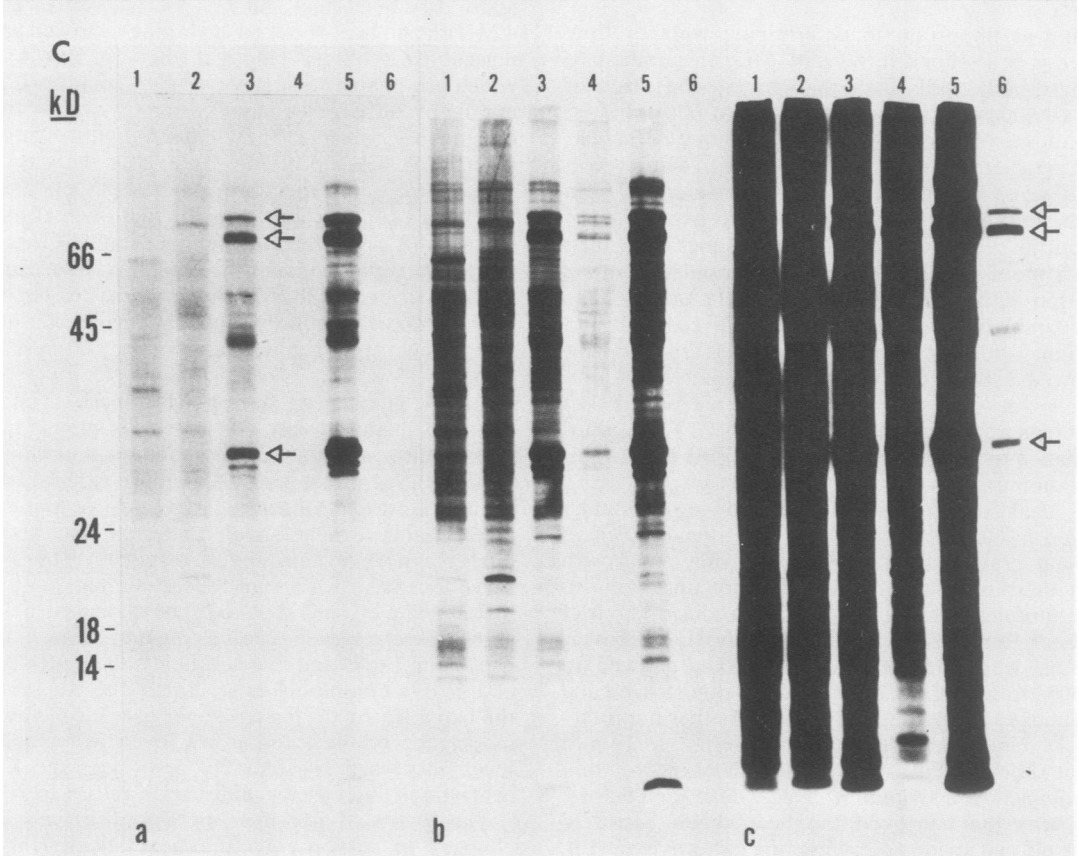
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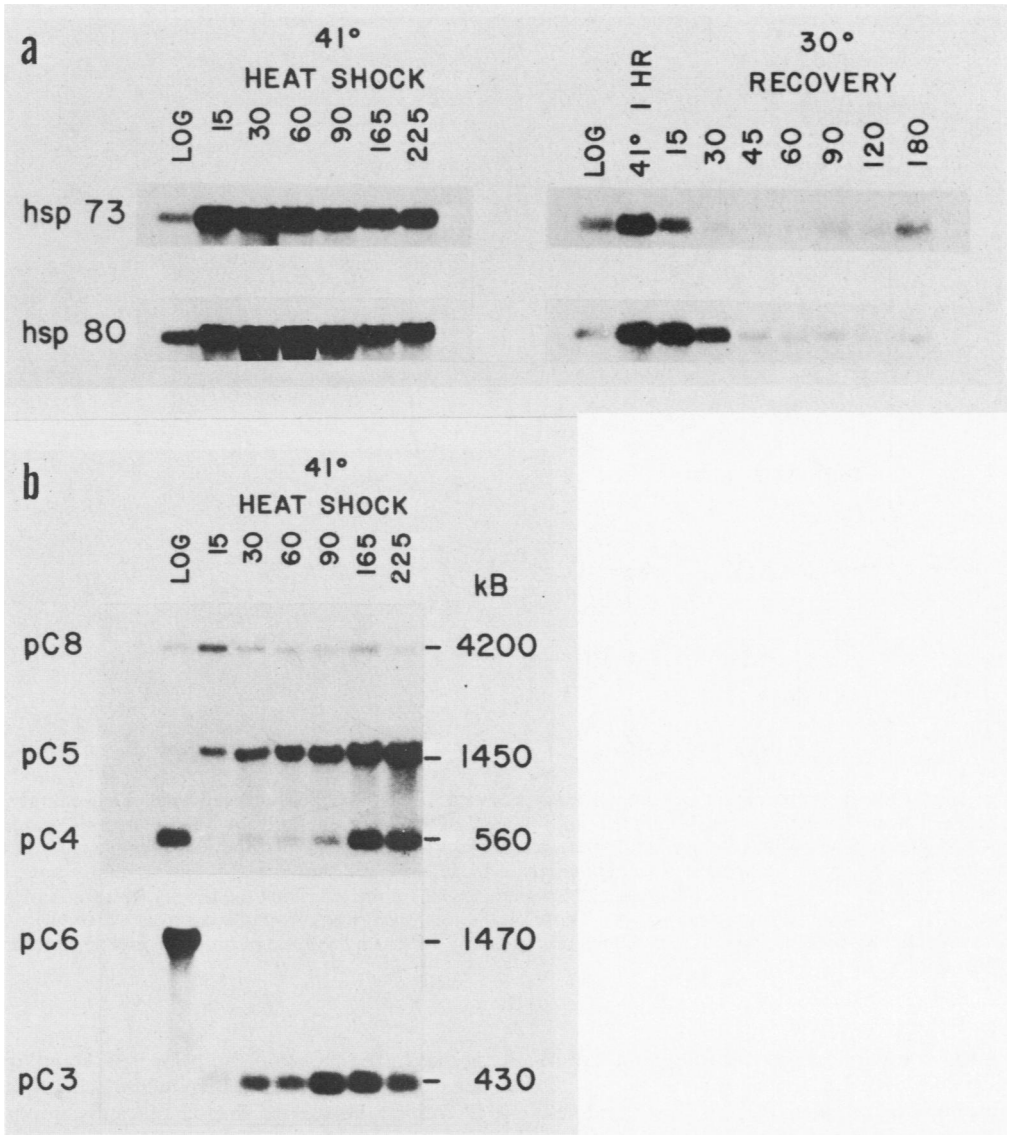

FIG. 3. Changes in cellular levels of mRNAs for hsp's and non-hsp's during the course of a continuous heat shock. Cells in log growth (80,000/ml) at 30°C were transferred to 41°C, and samples of cells were collected at various times during the heat shock (values are given in minutes). Total cellular RNA was extracted, separated on 1% agarose gels, transferred to membrane filters, and hybridized with ^{32}P-labeled plasmids containing genomic DNA inserts (see text) for either hsp73 or hsp80 mRNA (a) or cDNA inserts of five different mRNAs (b), and autoradiograms were prepared. Two to four plasmids were hybridized to each separate filter. The clones for hsp73 and hsp80 hybridized to mRNAs of 2,600 and 3,000 bp, respectively. To study recovery, cells in early log growth at 30°C were shifted to 41°C and left for 1 h. The cultures were then shifted back to 30°C and cells were collected at various times. Their RNAs were analyzed as described for the induction analysis. The numbers given indicate the time in minutes in recovery from heat shock. Only the data for the hsp mRNAs are shown. kB, kilobases.

FIG. 2. Changing rates and patterns of amino acid incorporation in short-starved, heat-shocked cells. Early log cells were collected and suspended in either 10 (a) or 60 (b) mM Tris at 30°C. After 1 h of starvation, one-half of each culture was transferred to 41°C. At various times during the starvation and subsequent heat shock, samples of cells were pulse-labeled with [^{3}H]lysine for 10 min. Triplicate samples were collected, and the incorporation of labeled amino acids into an acid-insoluble form was determined. The average value obtained for each time point was compared with the value for the incorporations during the first 10 min of starvation (in the respective medium) and expressed as a fraction of this. The closed circles (●) indicate the labeling pattern of cells starved at 30°C. The arrow indicates the time the heat shock was administered, and the open circles (○) indicate the amino acid incorporation values in cells at 41°C. At selected times samples of cells which had been pulse-labeled for 10 min were collected, and the total cell proteins from equal numbers of cells were separated on gradient SDS-polyacrylamide gels and subsequently fluorographed (c). Cells were collected at the following times: (1) during the last 10 min of the 1-h starvation in 10 mM Tris; (2) during the last 10 min of the 1-h starvation in 60 mM Tris; (3) during the first 10 min of the 41°C heat shock in 10 mM Tris; (4) during the first 10 min of the 41°C heat shock in 60 mM Tris; (5) during the second 10 min of the 41°C heat shock in 10 mM Tris; (6) during the second 10 min of the 41°C heat shock in 60 mM Tris. These time intervals are indicated by the numbers in panels a and b. Fluorograms were produced from different exposures of the dried gel: (a) 6 h; (b) 24 h; (c) 10 days. The arrows indicate the major heat shock proteins: hsp80, hsp73, and hsp34, the latter of which is a composite of several polypeptides (6, 27).

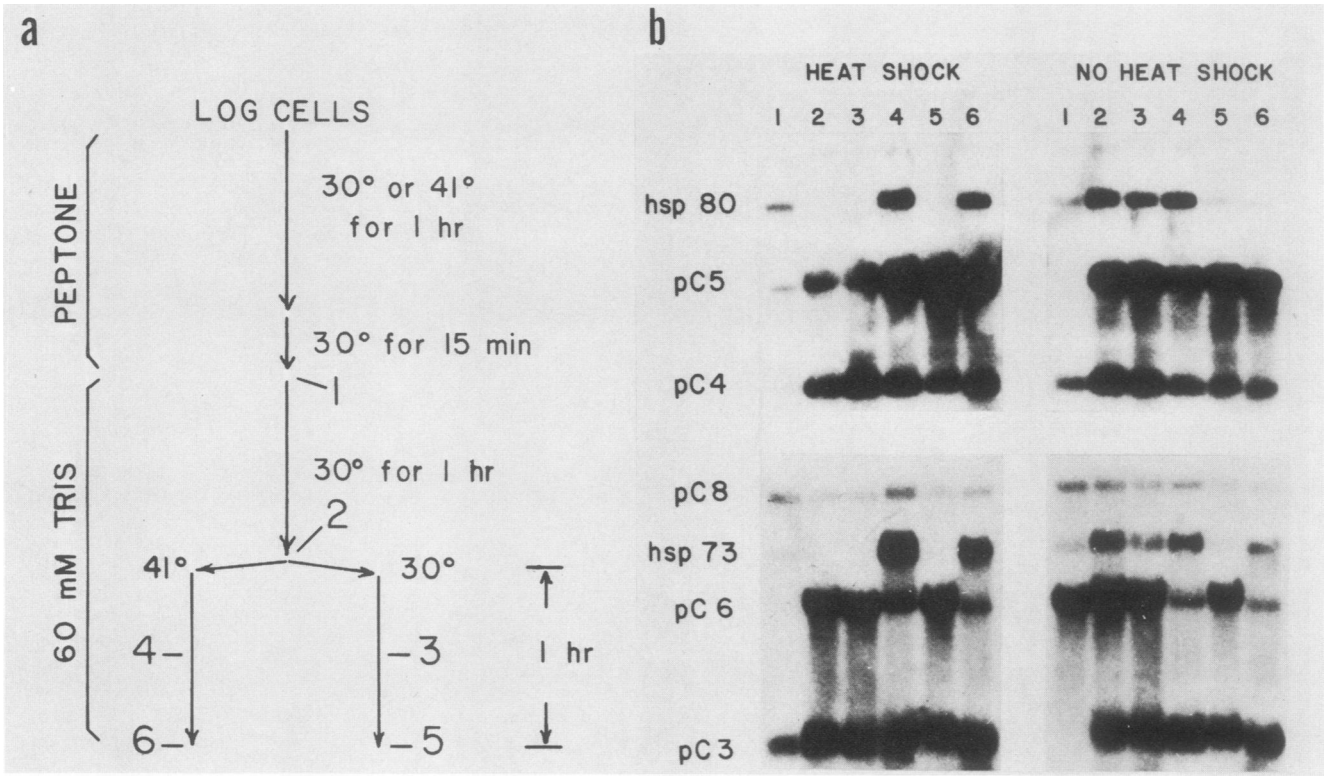

FIG. 4. Effects of a heat shock pretreatment on cellular mRNA levels in heat-shocked, starved cells. Cell cultures were treated as diagrammed in (a), and cells were collected at the designated times (lanes 1 to 6). Total cellular RNA was isolated and processed as indicated in the legend to Fig. 3. Duplicate filters were produced and each was probed with a different mixture of labeled plasmids homologous to both heat shock- and non-heat shock-specific sequences. Autoradiograms of those filters are shown in (b). The numbers above the lanes indicate the point in the diagram (a) when the cells were collected. The autoradiograms on the left show the results with RNA from cells which had been exposed to the 1-h, 41°C heat shock during log growth. Those on the right used RNA from the controls which had not received a heat shock while in growth medium. Direct comparison of the intensities shown by various hybridized plasmids is not appropriate since the specific activities of the probes differ.

served as a basis for comparison for the following experiments.

We next examined the metabolism of heat shock and non-heat shock mRNAs in 1-h-starved cells, those vulnerable to heat shock killing, and compared it with that seen in cells resistant to killing by this treatment under the same environmental conditions. Thus, the standard of comparison for the vulnerable cells would be cells in growth medium which had been transferred into starvation medium for 60 min and then heat shocked but which previously had been subjected to a 60-min heat shock 75 min before being transferred to starvation medium. Such a pretreatment allows an approximately 70 to 90% survival rate of these cells (Table 2) in contrast to the <0.01% survival rate of non-pre-heat-shocked cells. A diagram of the experimental protocol is given in Fig. 4a. Cells were treated and collected at the indicated times, and their total cellular RNA was isolated, separated by agarose gel electrophoresis, transferred to membrane filters, and hybridized with either the heat shock or non-heat shock probes. The resulting autoradiograms from these experiments are shown in Fig. 4b. From the point of view of non-heat shock messages, there are no obvious differences in mRNA metabolism during the heat shock in those cells which were going to survive as compared with those which were going to die. There are differences in the two cell populations in the amounts of all of these messages just before the cells were washed into starvation media, but this is due to the fact that the prior heat shock had caused some messages to increase in level while bringing about a decrease in others. However, during the subsequent starvation and heat shock treatments these differences were abolished.

The changes in abundance levels of the heat shock messages were clearly not the same in the two cell populations. However, it should be noted that a comparison of the levels of both hsp73 and hsp80 mRNAs in cells which had been starved for 60 min and then heat shocked for 30 min (Fig. 4b, lane 4) indicated that, although there might be more heat shock message in cells that had been previously heat shocked, the non-pretreated cells had a significant level of these sequences within them relative to their pretreated counterparts (ca. 80% for hsp80 mRNA and ca. 20% for hsp73 mRNA). These non-pretreated cells are the ones in which we previously could detect no protein synthesis after 30 min (see Fig. 2), and these cells, when transferred to 30°C after 90 min of heat shock, showed complete inviability. Thus, the reason these cells make so little hsp is not primarily due to a reduction in abundance of heat shock messages. Whether all the sequences we detect represent functional messages cannot be determined from these data.

Other differences were also seen in the heat shock messages when the two cell populations were compared. Surprisingly, a 1-h starvation itself induced elevated levels of both hsp73 and hsp80 mRNAs (Fig. 4, lane 2, right panels), and by 2 h the concentration decreased to the prestarvation

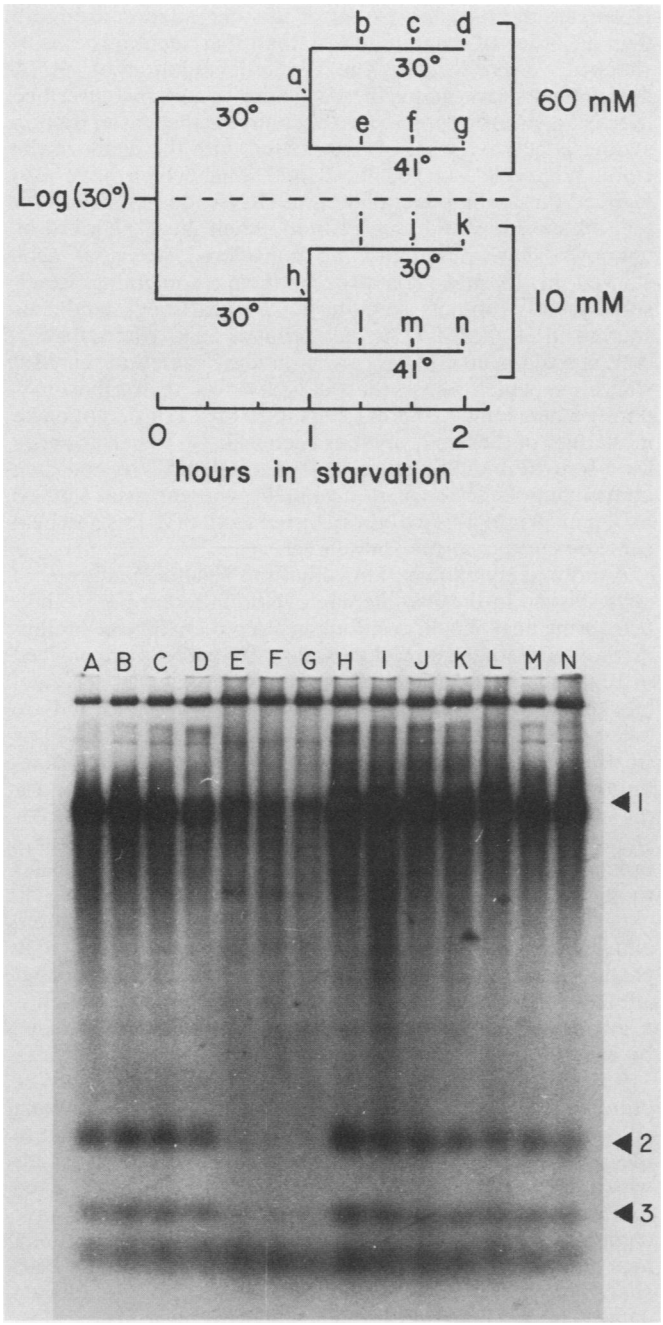

FIG. 5. Electrophoretic patterns of total cellular RNAs from cells heat shocked during starvation in either 10 or 60 mM Tris. Log cells were collected and suspended in either 60 (lanes a to g) or 10 (lanes h to n) mM Tris and allowed to starve for 1 h (lanes a and h) at 30°C. The two cultures were then each divided in half: one-half was shifted to 41°C (lanes e to g and l to n); the other remained at 30°C (lanes b to d and i to k). Cells were collected at 20 (lanes b, e, i, l), 40 (lanes c, f, j, m) and 60 (lanes d, g, k, n) min after the cultures were split. The line drawing indicates the experimental protocol in diagrammatic form. Total cellular RNA extracts were prepared, but before running the RNA samples they were heated to 60°C for 5 min to allow release of the 5.8S rRNA from the 26S rRNA. This treatment also causes the 26S rRNA to dissociate into two approximately equal fragments which are about the same size as the 17S rRNA because of the presence a "hidden" endonucleolytic cut which is introduced into the 26S rRNA about 10 to 15 min after it has entered the cytoplasm (5). Total RNA extracts were run on 2.2% agarose gels and stained with ethidium bromide. The arrowheads indicate the species of RNA which decrease in the 60 mM-starved, heat-shocked cells. From the electrophoretic analyses of RNA isolated from purified ribosomes (data not shown) the bands were identified as: 26S and 17S rRNA (1); 5.8S rRNA (2); 5S rRNA (3).

level (data not shown). If the cells had been previously heat shocked, then during the starvation period there was no detectable mRNA present of either variety. This finding is not totally surprising in that hsp's are present in the cell at >90% of their fully induced level as a result of the prior heat shock (K. Kraus, unpublished data), and it is possible that *T. thermophila* hsp's may regulate, by some feedback mechanism, the levels of their own mRNAs, as has been suggested for *Drosophila* sp. (4). There was a rapid reappearance of heat shock message at high abundance levels in the previously heat-shocked cells. However, in the cells without a pretreatment, a heat shock reduced the hsp73 and hsp80 message levels. However, the extent of this decline seems insufficient to account for the degree of loss of hsp synthesis.

Loss of rRNA during a heat shock of starved cells. The data thus far indicated that the reason protein synthesis declined in heat-shocked, 1-h-starved cells was primarily due to a translational lesion. Total cellular RNA isolated from these cells during the heat shock showed a measurable decline in amount (data not shown), which was not apparent in cells that were immune to the heat shock killing. As the bulk of the RNA in the cell is rRNA, we examined the overall pattern of RNA in the cell during the course of the heat shock. After electrophoresis on agarose gels, the ethidium bromide staining pattern of RNA from cells starved and heat shocked in 60 mM Tris starvation buffer (lethal) was compared with that obtained from cells starved and heat shocked in 10 mM Tris starvation medium (nonlethal). The results of such an experiment are shown in Fig. 5. Cells in 10 mM Tris, whether just starved or starved and heat shocked, showed no obvious changes in their RNA profiles during the course of the experiment. In distinct contrast, cells in 60 mM Tris, when heat shocked, displayed a loss of certain stainable RNA species which were rRNAs.

As a final demonstration that a crucial difference between cells which survive heat shock and those that do not was ribosome stability, we examined the stability of RNA in cells heat shocked while in 60 mM Tris but which had been exposed to a prior heat shock. The results in Fig. 6 show that cells which survive the heat shock have stable rRNA whereas in those destined to die there is a rapid and specific degradation of rRNA (Fig. 5). Although we have not quantitated the rate of loss of rRNA in these heat-sensitive cells, its decline roughly approximates the rate of decline in protein synthesis rate which we had previously observed (Fig. 2). Thus, it appears that a probable cause of the inability of the cell to mount an effective heat shock response shortly after transfer to 60 mM Tris is due to heat-induced degradation of rRNA, and consequently ribosome inactivation.

DISCUSSION

The unexpected finding of this work is that, under a rather narrow set of environmental conditions, *T. thermophila* cells can be killed by what should otherwise be a nonlethal heat shock. In this case, the inability to mount an effective heat shock response appears to be a general breakdown in protein-synthetic capacity rather than a specific loss in the ability to synthesize hsp's. Of course, the loss of the former results in the loss of the latter and, presumably, accounts for

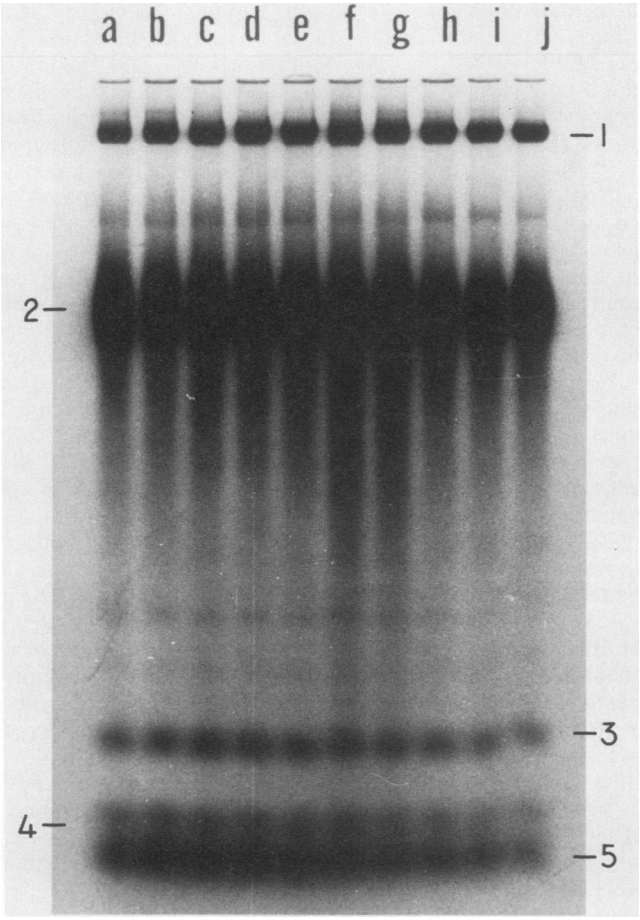

FIG. 6. Lack of rRNA degradation in cells heat shocked in 60 mM Tris which had been previously heat shocked while in log growth. Cells in log-phase growth (ca. 50,000/ml) at 30°C were transferred to 41°C for 1 h. They were then returned to 30°C for 30 min, after which they were washed into 60 mM Tris. The culture was then divided in half: one-half was left at 30°C and the other was shifted to 41°C. Samples were taken at this time (lanes a and f) and at 15 (lanes b and g), 30 (lanes c and h), 45 (lanes d and i), and 60 (lanes e and j) min. (Lanes a to e) cells kept at 30°C; (lanes f to j) cells heat shocked at 41°C). The numbers indicate the following: 1, DNA; 2, the large rRNAs; 3, 5.8S rRNA; 4, 5S rRNA; 5, tRNA.

the cells' demise. That *T. thermophila* cells can be killed at 41°C if protein synthesis is inhibited supports this contention.

The only other known stage in the life history of organisms during which an effective heat shock response cannot be mounted is found during early development in *Drosophila* sp. (10), sea urchins (23), and *Xenopus* sp. (3). In these organisms a heat shock administered shortly after fertilization is lethal. Whether there is any relationship between the lethalities in these two situations is not known.

Degradation of rRNA. The data presented herein show that, under a situation where mRNA metabolism for both hsp's and non-hsp's is apparently normal, rRNA can be preferentially degraded. Heat-stressed *Staphylococcus aureus* also show rRNA degradation (15), but in this case other RNAs are degraded as well. The induction of rRNA degradation in *T. thermophila* is a normal response in cells washed from growth medium into any starvation medium or in cells which have entered the late log phase of growth (27). However, the rate and extent of this degradation are more than an order of magnitude less than that seen in the heat-shocked, starved cells. The rapid degradation of rRNA during heat shock under the particular conditions described here is apparently sufficient to account for the loss in protein synthesis activity which is associated with the death of the cells. Why cells starved in 10 mM Tris, which have also induced the normal degradation of rRNA, do not also show the increased, rapid degradation when heat shocked is unknown. One possibility we considered was that cells starved in 10 mM Tris are producing something which subsequently protects them during the heat shock, and cells starved in 60 mM Tris do not produce this. Alternatively, cells starved in 60 mM Tris are producing something early in starvation which allows destabilization of their ribosomes during a heat shock whereas cells in 10 mM Tris do not make it. Neither of these possibilities seems likely, as cells starved for 1 h in 10 mM Tris when shifted to 60 mM Tris and then immediately heat shocked are killed, whereas cells starved for 1 h in 60 mM Tris and then shifted to 10 mM Tris and heat shocked survive at the 100% level.

A more likely explanation is that the plasma membrane of cells starved in the two media develop different permeabilities during heat shock, resulting in altered ion fluxes. Preliminary experiments support this idea. When cells are starved in 10 mM Tris and treated with Amiloride, an Na^+ channel blocker (2), they become sensitive to the heat shock and are killed in an identical fashion to those starved in 60 mM Tris (R. L. Hallberg, unpublished data). They also show degradation of their rRNA. This suggests a possible explanation for the cell death. An ionic imbalance in heat-shocked, 60 mM-starved cells exacerbates the normal ribosome degradative process, which normally begins immediately upon transfer to starvation conditions (27), to the point of destroying all capacity for protein synthesis. Later on during starvation, either the membrane properties are altered or, due to a change in the ribosome turnover rate, the cell is no longer affected. This explanation implies that rRNA degradation is a secondary event and that the immediate changes induced by heat shock are associated with membranes.

Ribosome modifications. In asking how rRNA can be preferentially degraded, it is important to consider what physiological treatments of the cell render them immune to the lethal effects of heat shock in 60 mM Tris. The cells which can survive are those which (i) have already starved for 15 h or more (in any starvation medium) or (ii) have entered a plateau state of growth, or (iii) have been recently heat shocked. One common metabolic similarity these cells share is that they modify their ribosomes (14; unpublished data). The modification brought about by heat shock most commonly identified in other systems is the dephosphorylation of ribosomal protein S-6 (8, 24). Our preliminary evidence gives no indication of a correlation between the state of ribosomal protein S-6 phosphorylation and the phenomenon we describe here. However, there are a number of other modifications of ribosomal proteins known to occur in cells undergoing physiological changes, and one of these may well be associated with the changes we report here. There is evidence (1, 26, 28) which points to the possibility, at least in *Drosophila* sp. and HeLa cells, that modification(s) of the protein-synthetic machinery occurs during a heat shock. Whether it is the ribosome which undergoes modification has not been determined.

How shifts in the ionic conditions of cells can, in some instances, lead to cell death after heat shock is not clear, nor is the mechanism whereby a prior heat shock protects these

same cells. It is clear, however, that ribosome degradation precedes cell death. It may be that hsp's interact with ribosomes or plasma membranes or both to confer protection. The relationship between the protection conferred by a prior heat shock and that conferred by the normal physiological changes of cells associated with an extended period of starvation, or with the transition from growth to a stationary phase, remains to be determined.

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