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Effect of Heat Shock on Ribosome Structure: Appearance of a New Ribosome-Associated Protein

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After a nonlethal but heat shock protein-inducing hyperthermic treatment, ribosomes isolated from *Tetrahymena thermophila* contained an additional 22-kilodalton protein (p22). When maximally ribosome associated, this protein was found to be on the small subunit in a 1:1 stoichiometric ratio with other ribosomal proteins. Using an antiserum directed against the purified 22-kilodalton protein, we found that non-heat-shocked and heat-shocked cells contain identical amounts of this protein, the only difference being that in the stressed cells p22 is entirely ribosome bound, whereas in the unstressed cells p22 has little or no detectable ribosome association. Because the two-dimensional electrophoretic properties of p22 showed no alterations after heat shock, this change in state of ribosome-p22 interaction does not appear to be caused by a chemical modification of p22. When not strongly ribosome associated, p22 is not found free in the cytoplasm. During that time in heat shock when p22 is first becoming ribosome associated, it is found preferentially on polysomal ribosomes. Subsequently, all ribosomes, whether polysome bound or not, obtain a bound p22. The functional significance of this association is discussed.

Many organisms respond to hyperthermal stress not only by inducing the synthesis of a specific set of proteins (heat shock proteins [hsp's]) but also by concomitantly suppressing the synthesis of normal cell proteins (non-heat-shock proteins [non-hsp's]; for a review, see reference 29). In some organisms, the suppression of non-hsp synthesis is mediated in large part at the translational level (3, 4, 30, 31, 33), whereas in others this appears to be more the result of transcriptional changes (23). In *Drosophila* (2, 19) and HeLa (16, 17, 33) cells, modification of both the initiation and elongation steps of protein synthesis appears to occur during the stress response. In *Drosophila* (8, 26), tomato (28), and HeLa (18) cells, the heat-induced functional alterations in the protein synthesis machinery correlate with a structural modification of one of the ribosomal proteins (r-proteins). Additionally, HeLa cells subjected to heat shock modify certain initiation factors which may be the cause of the inhibition of translation of non-hsp messages at the high temperature (6). Although suggestive of a link, no evidence directly links such structural modifications with the functional changes known to occur.

When *Tetrahymena* cells are subjected to heat shock, hsp synthesis is induced and non-hsp synthesis is repressed (7, 10, 13, 37). However, as with anaerobically growing yeast cells (24, 25), the heat shock response is transient, and as hsp synthesis declines, non-hsp synthesis returns. Evidence from our lab indicates that the repression of non-hsp synthesis during the early stages of heat shock, the subsequent decline of hsp synthesis, and the return of non-hsp synthesis later in the heat shock are, at least in part, regulated at the level of translation (13, 14; K. W. Kraus and R. L. Hallberg, submitted). For these reasons, we examined the ribosomes of cells at various times during heat shock to determine whether structural alterations which correlated with changes in translational events could be observed. We found that several changes occur in the array of tightly bound, ribosome-associated proteins during heat shock. One of these changes, the appearance of a 22-kilodalton (kDa) protein (p22), was characterized further. The kinetics of its association with the small ribosomal subunit during heat shock correlated positively with the return of non-hsp synthesis and with the decline in hsp synthesis. We measured the levels of this protein in heat-shocked and non-heat-shocked cells and determined the fraction of it which becomes tightly ribosome associated during these times. Our results indicate that this protein is always present in the cell and that heat shock causes the quantitative conversion of this protein from a non-ribosome-bound (or extremely weakly bound) state to one of high-affinity ribosomal interaction. When this first happens, p22 is initially found bound to functioning (i.e., polysomal) ribosomes. Finally, we attempted to ascertain what possible modifications account for the changes in its ribosome affinity.

MATERIALS AND METHODS

Culture conditions. A single strain of *Tetrahymena thermophila*, CU355 (IV), was used in all experiments. Cultures were grown at 30°C on a gyratory shaker in 1% Difco Proteose Peptone supplemented with 0.003% Sequestrene (CIBA-GEIGY Corp., Summit, N.J.). Heat shocks were carried out at 41°C in a shaking water bath as previously described (13, 14). Only cells in the early log phase of growth (less than 100,000 cells per ml) were used in these experiments. Cells were collected by centrifugation, washed once, repelleted, and stored frozen at −70°C.

The radioactive labeling of cellular proteins was accomplished by adding [^{3}H]lysine ([4,5-^{3}H]lysine; Amersham Corp., Arlington Heights, Ill.) at 20 μCi/ml to cultures of cells. This amino acid was used for labeling proteins because it is present in very low concentration in proteose peptone.

Ribosomal subunit isolation. Frozen cell pellets were thawed on ice and suspended in 4°C buffer containing 0.1 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.5) (LK buffer). Triton X-100 and deoxycholate were then added to a final concentration of 1.0 and 0.5%, respectively, the cells were ruptured with 10 to 15 strokes in a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N.J.), and the cell homogenate was centrifuged at 15,000 × *g* for 10 min at

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4°C. The supernatant solution was underlaid with a 2-ml, 15% sucrose pad containing 0.6 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.5) and then centrifuged at 100,000 × *g* for 8 h at 3°C. Ribosome pellets were rinsed carefully with H_2O and stored frozen at −70°C.

Ribosome pellets were suspended (usually to a concentration of 25 to 50 optical density units at 260 nm/ml) in a solution containing 0.6 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris (pH 7.5) (HK Buffer) and centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant (containing 50 to 70 optical density units at 260 nm) was then layered onto 15 to 35% linear sucrose gradients made up in HK buffer which was then centrifuged at 23,000 rpm in a Beckman SW27 rotor for 16 h at 3°C. After centrifugation, the gradients were fractionated into 2-ml portions, and the optical density at 260 nm of each was determined. Appropriate fractions containing the large and small subunits were pooled and precipitated with 2 volumes of 95% ethanol. The protease inhibitors aprotinin and phenylmethylsulfonyl fluoride were used at concentrations of 20 μg/ml and 2 mM, respectively.

To isolate ribosomes by different stringencies of washing, frozen cells were disrupted in LK buffer or in buffer containing 0.01 M KCl, 0.01 M Tris, and 0.0015 M $MgCl_2$ (pH 7.5) (RS buffer). After centrifugation of the cell lysates at 10,000 × *g* for 10 min, each supernatant was underlaid with a 15% sucrose pad. For cells lysed in RS buffer, the sucrose pad contained RS buffer. For those lysed in LK buffer, the supernatants were underlaid with sucrose pads containing one of the following: (i) LK buffer, (ii) HK buffer, or (iii) 0.8 M KCl–0.01 M Tris–0.01 M $MgCl_2$ (pH 7.5). These tubes were then centrifuged at 100,000 × *g* for 5 h at 3°C. The resulting washed ribosome pellets were prepared for gel electrophoresis as described below.

Monosome and polysome isolation. Approximately 20 × 10^6 cells from an appropriately treated culture were collected by centrifugation, ruptured by vortexing in 3 ml of polysome lysis buffer (100 mM KCl, 20 mM $MgCl_2$, 20 mM Tris, 2% spermidine, 1.0 mg of heparin sulfate per ml, 2 mM dithiothreitol, 0.05% Nonidet P-40 [pH 7.5]), and centrifuged at 12,000 × *g* for 10 min. The resulting supernatant was layered on a 15 to 45% linear sucrose gradient containing 100 mM KCl, 20 mM $MgCl_2$, 20 mM Tris chloride, and 2 mM dithiothreitol (pH 7.5) and centrifuged at 23,000 rpm in an SW27 rotor for 4 h at 6°C. The gradient was fractionated into 2-ml portions, the A_{260} of each fraction was determined, and the fractions, containing either polysomes or monosomes, were pooled. The ribosomes in each of these were precipitated with ethanol as described above.

Gel electrophoresis of r-proteins. Ethanol-precipitated ribosomal subunits were redissolved in H_2O. The A_{260} of the solution was measured, and from this measurement the concentration of r-proteins was determined (12). The samples were diluted with an equal volume of 2× Laemmli sample buffer (20) and heated to 100°C for 2 min. In some cases, the r-proteins were extracted from purified ribosomes by the acetic acid extraction method of Gorenstein and Warner (9). The 22-kDa protein, p22, was solubilized by this procedure, as could be seen when such extracted proteins were subsequently dissolved in Laemmli sample buffer and run on sodium dodecyl sulfate (SDS)-containing gels (20). However, the 22-kDa protein was not found to migrate either anodally or cathodally when we attempted to separate these same purified proteins by the two-dimensional electrophoretic procedure of Lastick and McConkey (22). This explains why the 22-kDa protein was not seen in our previous work (15).

The procedure for the one-dimensional separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been described previously (13, 14). Two-dimensional gel electrophoresis of proteins was performed by the procedures of Guttman et al. (11). The first dimension was nonequilibrium pH gradient electrophoresis run for 2,400 V · h. The second dimension was 15% SDS-PAGE run for 800 V · h. Both one- and two-dimensional gels were stained and destained as previously described (13, 14). The fluorographic procedure of Lasky and Mills (21) was used to visualize radioactively labeled proteins.

Quantitation of stained protein bands in one-dimensional SDS gels was accomplished by scanning the lanes of gels with an LKB ultrascan laser densitometer (LKB Instruments, Inc., Gaithersburg, Md.). Chart recorder tracings of such scans were copied onto heavy paper, and the appropriate peaks were cut out and weighed. Comparable quantitative results were obtained by using either Coomassie brilliant blue- or acid-fast-green-stained gels.

Preparation of 22-kDa protein antiserum. The 22-kDa protein, p22, was purified by excising the appropriate band from lightly stained one-dimensional SDS-polyacrylamide gels in which small ribosomal subunit proteins had been separated. The protein was electroeluted from the macerated gel pieces directly into dialysis tubing. The eluted material was dialyzed overnight at 4°C against buffer containing 10 mM KCl and 10 mM Tris (pH 7.5). The dialyzed protein solution was partially lyophilized to reduce its volume and subsequently emulsified with 3 volumes of Freund complete adjuvant. To obtain antibodies, rabbits were injected with the purified protein by the procedure of Vaitukaitis (35). Approximately 40 μg of protein was injected in 50-μl aliquots intradermally at several sites (approximately 20 to 30) around the backs of two rabbits. The same injection procedure was repeated 9 weeks later. At 1 week after the booster injections, both rabbits were bled from the ear. The serum was processed by standard procedures (5) to purify the immunoglobulin G fraction.

Immunoblotting. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, N.H.) by a modified version of the procedure of Towbin et al. (34). The transfer solution used contained 12.5 mM Tris, 96 mM glycine, 0.05% SDS, and 10% methanol. Transfers were typically carried out at 0.6 A for 2 h. Antigen-antibody reactions and the visualization of those complexes with peroxidase-coupled goat anti-rabbit serum were carried out exactly as described by Allis et al. (1).

To determine the relative amount of p22 in a particular sample, various amounts of that sample were electrophoresed, transferred, and visualized. The stained and still wet nitrocellulose filter was scanned with an LKB ultrascan laser densitometer. Chart recorder tracings of the scan were transferred to heavy paper, cut out, and weighed. By using low concentrations of r-proteins, the amount of anti-p22 visualized on the filters was found to be proportional to the amount of protein electrophoresed (see Fig. 6).

RESULTS

The proteins synthesized by *T. thermophila* during a 41°C heat shock and a recovery from heat shock are shown in Fig. 1. As reported previously (13, 37), the synthesis of hsp's with molecular masses of 73 and 80 kDa and of a group with molecular masses of about 30 kDa is detectable within the first few minutes of the temperature shift and dominates the

array of newly synthesized proteins. The synthesis of proteins in cells growing at 30°C was initially repressed during the 41°C heat treatment (Fig. 1, lanes 2 and 3) but gradually returned (lanes 4, 5, and 6). By 45 to 60 min after initiation of heat shock, the synthesis of many of the non-hsp's could already be seen. During the same period of time that non-hsp synthesis returned, the synthesis of the hsp's declined so that by 120 min at the heat shock temperature, the protein synthesis pattern was almost identical to that of the non-heat-shocked cells (Fig. 1, lanes 1 and 6). The levels of a number of specific non-hsp mRNAs were monitored throughout heat shock, and although some decreased during the initial stages of heat shock, others showed no decreases and some actually increased (13; Kraus and Hallberg, submitted). Those mRNA species that were retained initially dissociated from polysomes, but after about 1 h they again became polysome associated (Kraus and Hallberg, submitted). In addition, the decline in synthesis of hsp73 and hsp80

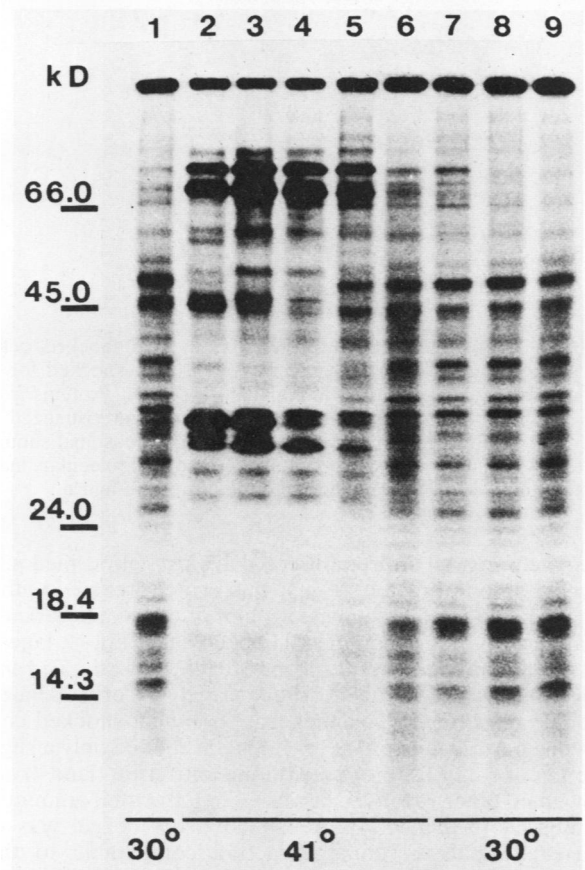

FIG. 1. Proteins synthesized during normal growth, heat shock, and recovery from heat shock. Cells growing at 30°C were shifted to 41°C, and at various times during heat shock or during recovery at 30°C from a 60-min heat shock aliquots of cells were pulse-labeled with [^{3}H]lysine for 15 min. Samples of total cell protein were prepared, separated by SDS-PAGE (17% from), and fluorographed as described in Materials and Methods. The times of labeling of the various samples were as follows. Lanes: 1, labeled for 15 min during early-log-phase growth at 30°C; 2, labeled from 0 to 15 min of heat shock; 3, labeled from 15 to 30 min of heat shock; 4, labeled from 30 to 45 min of heat shock; 5, labeled from 45 to 60 min of heat shock; 6, labeled from 105 to 120 min of heat shock; 7, labeled from 0 to 15 min of recovery from a 60-min heat shock; 8, labeled from 15 to 30 min of recovery; 9, labeled from 45 to 60 min of recovery. Molecular mass markers, in kilodaltons, are shown to the left of the figure.

did not occur with the same kinetics as the decrease in overall concentration of their respective mRNAs (13); after 1 h at 41°C, these mRNAs were not completely polysome associated, as they had been during the earliest stage of the heat shock (Kraus and Hallberg, submitted). These findings indicated to us that translational regulation of protein synthesis must be involved in controlling the array of proteins synthesized at different times during heat shock.

Since the ribosome is the major component of the translational apparatus, a change in its structure could reflect a change in the functional state of the protein synthesis machinery. For this reason, we examined the r-proteins isolated from *Tetrahymena* cells undergoing heat shock or recovering from heat shock to see if correlative changes could be found. The proteins from high-salt-concentration (0.6 M KCl)-washed ribosomes of cells which had been treated as described in the legend to Fig. 1 are shown in Fig. 2. The pattern of r-proteins was found to undergo several reproducible changes during heat shock which were reversed during the recovery period. These changes, as indicated by the arrows, included an increase in the staining intensity of a doublet at 38 kDa, the disappearance of a band at 34 kDa, the disappearance of several minor bands at 30 kDa, and the appearance of a band at 22 kDa. When protease inhibitors were included in all steps of the ribosome isolation procedure, we found them to have no effect on these changes in protein arrays, indicating that they were not the result of proteolytic artifacts (27). Additional evidence, presented below, confirms that proteolysis could have played no role in generating these results.

The appearance of the 22-kDa protein was examined in more detail because the kinetics of its appearance correlated reasonably well with the return of non-hsp synthesis and the decline of hsp synthesis during the heat shock. This protein was found to be associated with the small ribosomal subunit when purified ribosomes from cells heat shocked for 1 h were dissociated on a 0.6 M KCl-containing sucrose gradient (Fig. 3). Its association with the ribosome was both strong and specific under heat shock conditions. The stoichiometry of the 22-kDa protein (p22) relative to the other r-proteins was determined by comparing the staining intensity of the 22-kDa band (it is a single polypeptide; see Fig. 8) with the intensity of two other well-resolved bands at 52 and 12.5 kDa (Fig. 2). The electrophoretic profiles of several different concentrations of r-protein separated by one-dimensional SDS-PAGE and stained with two different dyes were scanned, and the relative amounts of stainable protein in the two bands were determined, as described in Materials and Methods. After correcting for size differences and the number of proteins per band, the 22-kDa p22 was found to be represented in the r-protein population at a concentration such that all (or virtually all) of the ribosomes in heat-shocked cells possessed an associated 22-kDa protein (that is, assuming one p22 per ribosome) (Table 1).

Because p22 appeared on the ribosome in response to heat shock, we determined whether the synthesis of this protein was also induced by the heat treatment. Ribosomes were isolated from cells which had been labeled with [^{3}H]lysine during a 60-min, 41°C heat shock. The 22-kDa protein which appeared on the ribosome as the result of this treatment was not radioactively labeled, indicating either that p22 contains no lysine residues or that it was not synthesized in response to heat shock. When we labeled cells for 3 h during early-log-phase growth at 30°C, chased them in fresh, nonradioactive medium for 60 min at 30°C, and then heat shocked them at 41°C for 60 min, the p22 protein found in the purified

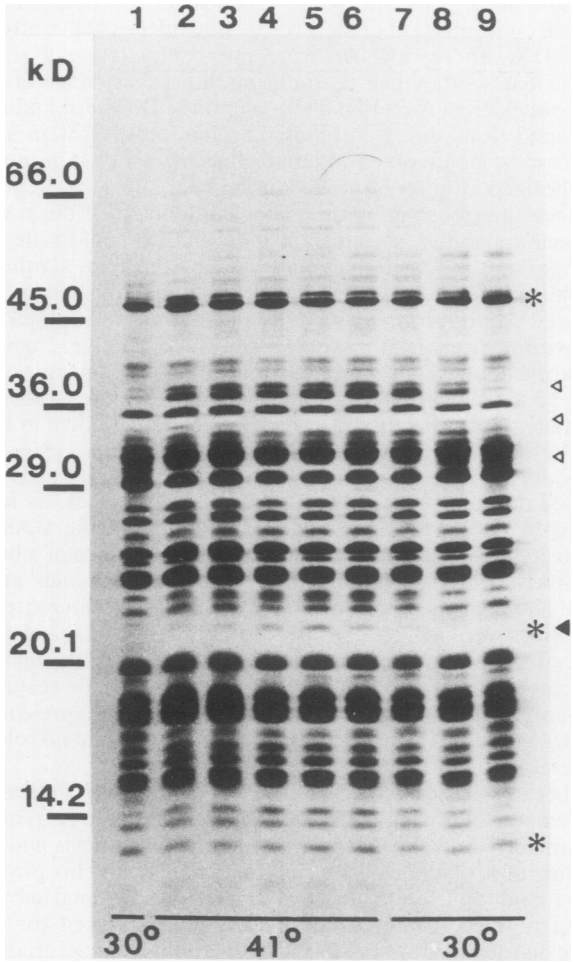

FIG. 2. R-proteins purified from cells experiencing normal growth, heat shock, and recovery from heat shock. Ribosomes were isolated from the same cells which had been radioactively labeled in Fig. 1. R-proteins were prepared, separated by SDS-PAGE (17%), and stained with Coomassie brilliant blue as described in Materials and Methods. The gel contains the following. Lanes: 1, r-proteins from non-heat-shocked cells; 2, r-proteins from cells transferred to 41°C for 15 min; 3, r-proteins from cells at 41°C for 30 min; 4, r-proteins from cells at 41°C for 45 min; 5, r-proteins from cells at 41°C for 60 min; 6, r-proteins from cells at 41°C for 120 min; 7, r-proteins from cells heat shocked for 60 min and then allowed to recover at 30°C for 15 min; 8, r-proteins from cells having recovered for 30 min; 9, r-proteins from cells having recovered for 60 min. The open triangles indicate reproducible changes seen in the staining patterns. The closed triangle indicates the 22-kDa protein which appears on ribosomes during heat shock. The asterisks indicate the protein bands used for quantitation in Table 1. Molecular mass markers, in kilodaltons, are shown to the left of the figure.

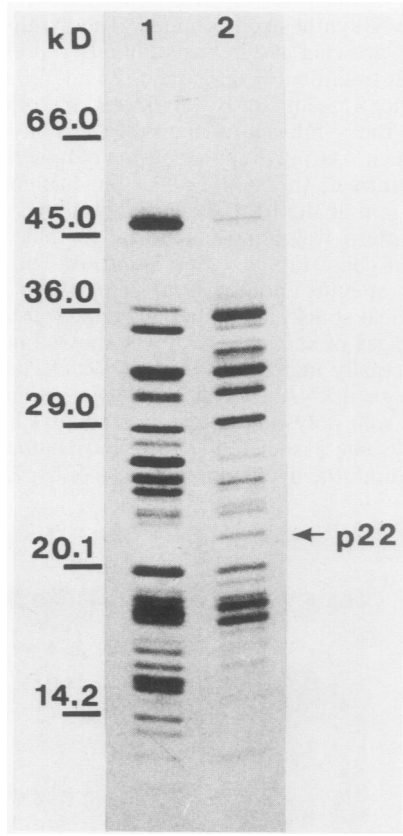

FIG. 3. Ribosomal subunit proteins from heat-shocked cells. Ribosomal subunits were prepared from cells heat shocked for 60 min at 41°C as described in Materials and Methods. Proteins from each subunit were electrophoretically separated and visualized as described in the legend to Fig. 2. Lanes: 1, large ribosomal subunit proteins; 2, small ribosomal subunit proteins. Molecular mass markers, in kilodaltons, are shown to the left of the figure.

ribosomes isolated from these cells was radioactively labeled to the same extent as all of the other r-proteins. Thus, the previous result could not have been due to the absence of lysine in this protein. More importantly, it showed that p22, either in some precursor form or in its mature form, was synthesized during normal growth at 30°C and that the 41°C treatment resulted in its conversion to a strong association with the ribosome.

To characterize p22 and any possible precursor or modified forms in further detail and to follow the metabolism of these proteins in both heat-shocked and non-heat-shocked cells, we prepared antisera directed against gel-purified p22. Immune serum directed against this protein reacted with a single 22-kDa band on a Western blot of electrophoretically separated r-proteins from heat-shocked cells (Fig. 4, lanes 1 and 3); preimmune serum did not react with any *Tetrahymena* proteins (lane 5). A similar analysis of equivalent amounts of purified ribosomes from non-heat-shocked cells showing no stainable p22 (Fig. 4, lane 2) elicited only a slight reaction of a 22-kDa protein with the antiserum (lane 4) and showed no other reactive species. When the total amount of the non-heat-shocked r-protein loaded on the gel was increased 20-fold, a staining reaction comparable to that shown with r-protein from heat-shocked cells was detected in the immuno-Western blot and again showed no other immunoreactive band. This result allowed us to rule out the possibility that p22 existed in an electrophoretically alternative form on ribosomes of non-heat-shocked cells. Additionally, it indicated the approximate difference in the concentration of p22 in the two r-protein samples.

The non-ribosome-associated p22 could exist in some precursor form and in response to heat shock could be modified to facilitate its association with the ribosome. To compare the form of all p22 immunologically related proteins in non-heat-shocked cells with those in heat-shocked cells, we probed Western blots of electrophoretically separated total cell protein extracts with our p22 antiserum (Fig. 5).

TABLE 1. Stoichiometry of ribosome-bound p22 as compared with other r-proteins[a]

Protein band (kDa)	Proteins/ band[b]	% of total r-protein mass[c]		Mass (kDa) of protein/band[d]		No. of proteins/ ribosome[e]	
		CBB[f]	AFG[g]	CBB	AFG	CBB	AFB
52	2 L	5.17	6.40	103.4	128.0	0.99	1.23
22 (p22)	1 S	1.11	0.88	22.2	17.6	1.01	0.80
12.5	1 S, 1 L	1.18	1.16	23.6	23.2	0.94	0.93

[a] Purified r-proteins from cells heat shocked for 1 h were separated by SDS-PAGE as described in the text. Duplicate gels were stained with either Coomassie brilliant blue or acid fast green. Both gels were scanned, and traces were plotted as described in the text. The areas under the entire traces (total r-protein), as well as under individual peaks at 52, 22, and 12.5 kDa, were determined as described in Materials and Methods.

[b] The number and location (large [L] or small [S] subunit) were determined from two-dimensional gels (15) and one-dimensional gels of purified subunit proteins (Fig. 3).

[c] (Area under a given peak/area of total r-protein) × 100.

[d] We assumed a mass of 2,000 kDa of protein per ribosome (12).

[e] We divided the estimated total mass of protein in the band by the size (in kilodaltons, as determined electrophoretically) of the protein in the band.

[f] CBB, Coomassie brilliant blue.

[g] AFG, Acid fast green.

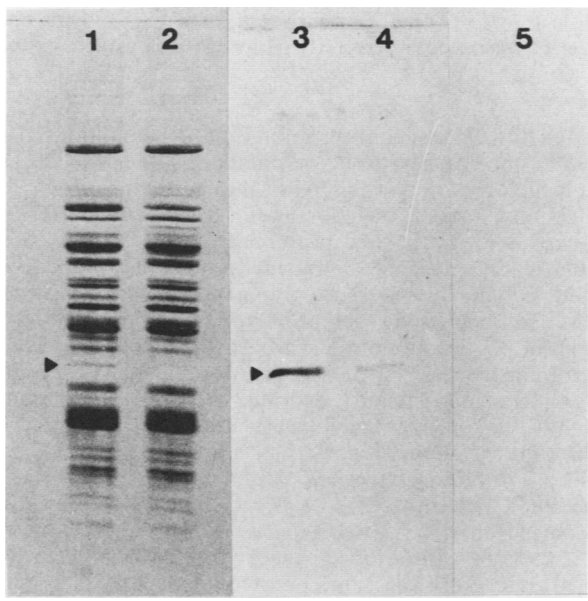

FIG. 4. Immunoblot analysis of electrophoretically separated r-proteins from non-heat-shocked and heat-shocked cells. Duplicate samples of purified r-proteins were separated by one-dimensional SDS-PAGE, either stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to nitrocellulose, and incubated with anti-p22 antiserum, and the immunoreactive r-proteins were visualized with a peroxidase-conjugated second antibody (lanes 3, 4, and 5) as described in Materials and Methods. R-proteins from non-heat-shocked cells are shown in lanes 2 and 4; r-proteins from cells heat shocked for 60 min are shown in lanes 1 and 3. The gel which was stained contained 25 μg of r-protein per lane, whereas the gel used for Western transfer and immunoblot analysis contained 2.5 μg of r-protein per lane. The lower r-protein amount electrophoresed for the Western blot was used to ensure that the reaction product obtained was proportional to the amount of r-protein present. An immuno-Western blot of electrophoretically separated total *Tetrahymena* r-protein (100,000 cell equivalents) reacted with preimmune serum is shown in lane 5 (this sample would contain about 25 μg of r-protein [12]).

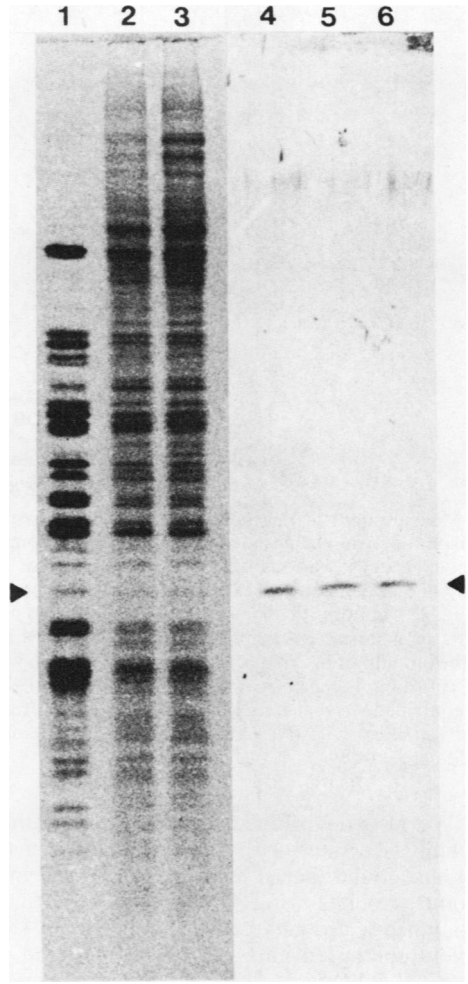

FIG. 5. Immuno-Western blot analysis of total cell protein from non-heat-shocked and heat-shocked cells. Proteins were separated by one-dimensional SDS-PAGE and stained with Coomassie brilliant blue (lanes 1, 2, and 3) or transferred to nitrocellulose and incubated with anti-p22 antiserum, and the immunoreactive proteins were visualized as described in the legend to Fig. 4 (lanes 4, 5, and 6). R-protein from cells heat shocked at 41°C for 60 min is shown in lanes 1 and 4. Total cell protein from non-heat-shocked cells is shown in lanes 2 and 5. Total cell protein from cells heat shocked for 60 min is shown in lanes 3 and 6. Lanes which were stained contained either 25 μg of r-protein or 50,000 cell equivalents of total cell protein. Lanes used for the Western blot analysis contained either 2.5 μg of r-protein or 5,000 cell equivalents of total cell protein. The arrows indicate the position of p22.

For both heat-shocked and non-heat-shocked cell extracts, the antiserum reacted only with a single 22-kDa band on the Western blot, indicating that p22 does not undergo a detectable size alteration during heat shock. Whether 2-mercaptoethanol was used to reduce the proteins before electrophoresis also had no effect on the electrophoretic mobility of the immunoreactive species. These results also provide additional evidence that the appearance of p22 on the ribosome cannot be caused by proteolysis.

Using Western blots similar to those shown in Fig. 4 and 5 to make quantitative measurements of the relative concentration of p22 in the two total cell protein samples, we determined that the amount of this protein in growing cells and in cells heat shocked at 41°C for 1 h is indistinguishable

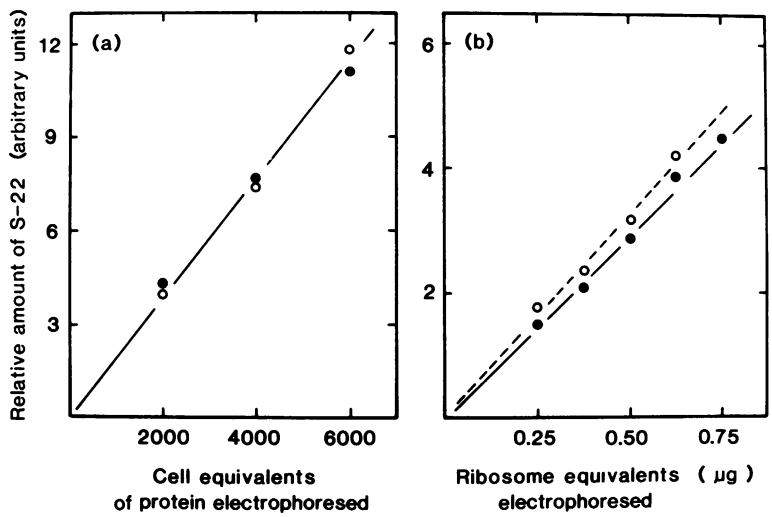

FIG. 6. Measurement of the amount of p22 in total cell protein extracts from non-heat-shocked and heat-shocked cells and in purified r-proteins isolated from heat-shocked cells. (a) To compare the relative amount of p22 in total cell proteins from heat-shocked and non-heat-shocked cells, various quantities of extracts of non-heat-shocked cells (●) and cells heat shocked for 60 min at 41°C (○) were separated by one-dimensional SDS-PAGE, transferred to nitrocellulose, reacted with anti-p22 antiserum, and visualized as described in the legend to Fig. 4. Quantitative densitometric scans of the immunoblots (see Materials and Methods) were made to obtain the values plotted. The amounts of material electrophoresed are expressed in terms of cell equivalents (1,000 cells contain 1.25 μg of protein [12]). (b) To determine the amount of p22 in a heat-shocked cell which was ribosome associated, various amounts of total cell extracts of cells heat shocked for 60 min (○) containing known amounts of r-protein (20% of the protein in *T. thermophila* is r-protein [12]) and amounts of purified ribosomes equivalent to that found in those cells (●) were electrophoresed, visualized, and quantitated as described for panel a. The values on the abscissa are expressed in terms of the amount of r-protein in the sample electrophoresed, whether in a purified form or as a component of the whole cell extract.

(Fig. 6a). We also determined the relative amount of p22 which became associated with the ribosomes after a 60-min heat shock and found that at least 90% of all p22 in the cell was ribosome associated (Fig. 6b). Therefore, virtually all of the p22 found in a growing cell can, upon heat shock, be quantitatively converted into a strong association with the small ribosomal subunit.

To examine further the form of p22 in non-heat-shocked cells, we looked for charge differences in the protein before and after heat shock. Total cell proteins from non-heat-shocked and from heat-shocked cells were separated by two-dimensional gel electrophoresis, transferred to nitrocellulose, and probed with the antiserum. These immuno-Western blots (Fig. 7) revealed no change in the electrophoretic properties of p22, indicating that it did not undergo a charge modification during heat shock. Unless some other modification of p22 occurs in response to heat shock that has no effect on its overall charge and size as determined by gel electrophoresis, which we cannot rule out, the reason that p22 becomes tightly ribosome associated must be caused by a change in the structure of the ribosome or a change in some component which reacts with it.

One possible explanation for our results might be that p22 is a normal ribosomal structural protein which is always ribosome associated but which is tightly bound only as the result of a heat-shock-induced modification of some ribosome-associated component. If p22 is weakly ribosome associated in non-heat-shocked cells, then the high salt concentrations used for ribosome purification might cause the dissociation of this protein during ribosome isolation. If so, lowering the stringency of the isolation procedure might allow a weakly bound p22 to remain ribosome associated. To investigate this possibility, we prepared ribosomes from heat-shocked and non-heat-shocked cells with very low (0.01 M KCl), low (0.1 M KCl), high (0.6 M KCl), and abnormally high (0.8 M KCl) salt concentration isolation conditions. The low salt concentration isolation conditions (0.1 M KCl) are those which we know retain all of the factors necessary for in vitro polypeptide elongation and ribosome runoff (32). As was expected, the stained proteins from the various ribosome isolates revealed a considerably larger number of stained protein bands in those prepared with the 0.01 M and 0.1 M KCl-containing solutions (data not shown). The amount of p22 in each purified ribosome sample was determined by using the anti-p22 serum to probe Western transfers of electrophoretically separated r-proteins in amounts known to yield an immunological staining reaction proportional to the amount of r-protein present (Fig. 8). No significant differences were apparent in the amount of p22 associated with 0.01, 0.1, 0.6, or 0.8 M KCl-washed ribosomes from non-heat-shocked cells. Also, the amount of p22 associated with ribosomes of heat-shocked cells remained essentially the same. We conclude that either the p22-ribosome interaction in non-heat-shocked cells is so weak that it cannot survive centrifugation under the lowest stringency isolation conditions or, alternatively, that p22 is not ribosome associated in non-heat-shocked cells. In either case, the heat shock must induce some change, or changes, which bring about the quantitative conversion of p22 to a ribosome interaction which survives the most stringent isolation conditions.

If the appearance of tightly bound p22 signals a ribosome which is in some way functionally altered, then one might reasonably expect that the first ribosomes with a strongly associated p22 should be found preferentially localized in the pool of either active or inactive ribosomes. To test this idea, we separated monosomes and polysomes from cells at two times after a transfer to heat shock (41°C) conditions (Fig. 9). As would be expected if p22 was a unit r-protein, when p22 became fully ribosome associated after 60 min of heat shock,

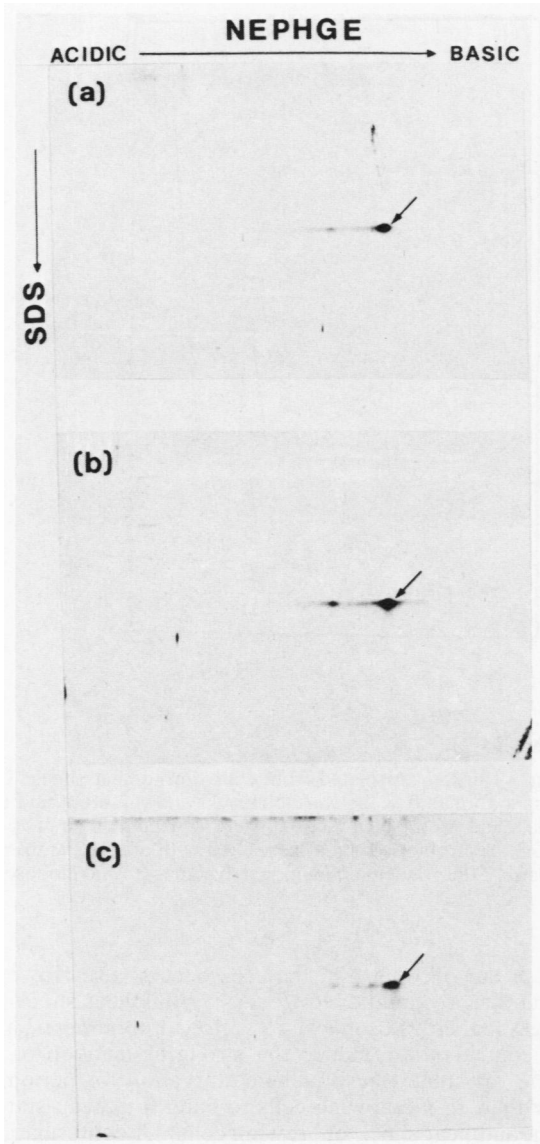

FIG. 7. Immuno-Western blot analysis of total cellular protein separated by two-dimensional gel electrophoresis. Total cellular protein was separated electrophoretically in the first dimension by nonequilibrium pH gradient electrophoresis and in the second dimension by SDS-PAGE as described in Materials and Methods. Proteins were transferred to nitrocellulose and immunoreacted with the anti-p22 antiserum as described in the legend to Fig. 4. One major polypeptide was detected by anti-p22, as indicated by the arrows. The samples run were from (a) non-heat-shocked cells, (b) cells heat shocked for 60 min at 41°C, and (c) an equal mixture of the non-heat-shocked and heat-shocked cell extracts.

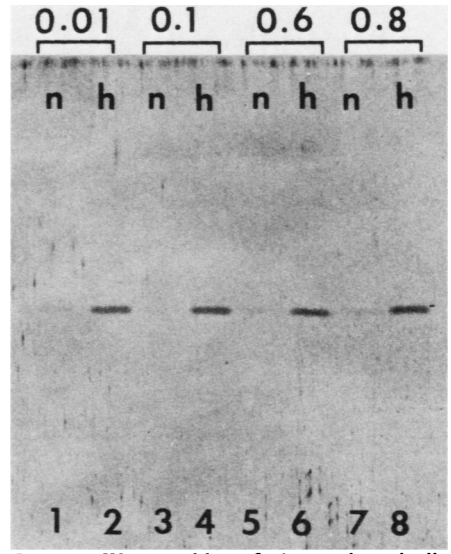

FIG. 8. Immuno-Western blot of electrophoretically separated r-proteins from ribosomes isolated under different stringencies. Ribosomes were centrifuged from cell lysates through sucrose pads containing different concentrations of KCl (indicated in molarities above the individual lanes) as described in Materials and Methods. Ribosomes were prepared from non-heat-shocked cells (n) and from cells heat shocked for 60 min at 41°C (h). Equal quantities of r-proteins were electrophoresed, and immuno-Western blot analysis of these r-proteins with anti-p22 antiserum was performed as described in Materials and Methods.

it was distributed equivalently among the polysomal and monosomal ribosomes of the cell (Fig. 9b). However, after 15 min of heat shock, when only partial association had occurred, p22 was found to be preferentially associated only with the polysomal ribosomes (Fig. 9a). Polysomal ribosomes comprise only about 20% of the total ribosomes in the cell at this time; this indicates the preferential utilization of ribosomes with a tightly bound p22 for protein synthesis at a time when the translational properties of the cells are undergoing changes. Whether there is any direct relationship between these two phenomena remains to be rigorously tested.

Finally, as can be seen in Fig. 9, no immunoreactive material was found at the top of either of the gradients. All of p22 is ribosome bound after 60 min of heat shock; this result is not surprising for these cells. However, after 15 min at 41°C, less than 25% of p22 is strongly ribosome associated (data not shown). The remainder of the 22-kDa protein in these cells was accounted for in the pellet of the centrifuged crude homogenate, the supernatant of which was spun on the sucrose gradient. What the 22-kDa protein interacts with which allows it to be pelleted at such a relatively low *g* force remains to be determined.

DISCUSSION

Ribosomes isolated from *T. thermophila* cells heat shocked at 41°C contain an additional protein, p22, strongly bound to the small ribosomal subunit. By some criteria, this protein resembles an r-protein which may be exchangeable. Its cellular concentration relative to other r-proteins is equimolar, and its distribution throughout the ribosome population is uniform when it is fully ribosome associated. The fact that we were unable to find little association of p22 with ribosomes from nonstressed cells under the least stringent washing conditions raises the distinct possibility that it may have no ribosome interaction at all in these cells. However, because p22, when not ribosome bound, is apparently not freely cytoplasmic, it may be that in nonstressed cells p22 is strongly associated with cytoplasmic structures yet still interacting with ribosomes, albeit in a less tenacious fashion. The resolution of the question of the cellular site of p22 during normal growth will require some sort of in situ

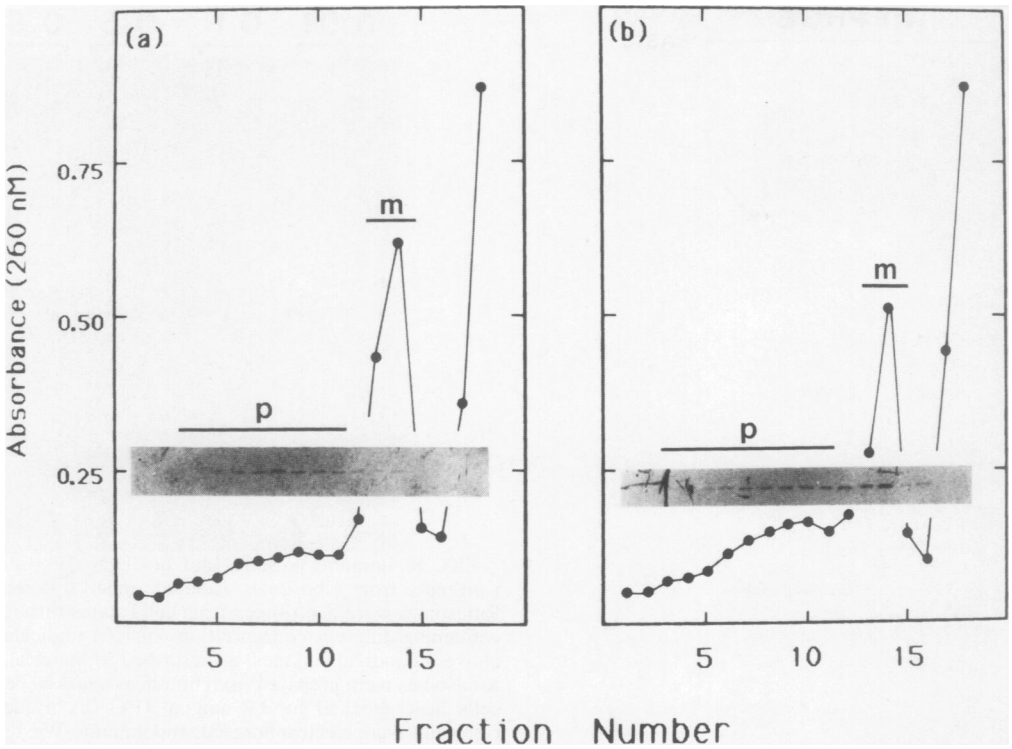

FIG. 9. Determination of the distribution of p22 in monosomal and polysomal ribosomes isolated from cells during heat shock. Cells growing at 30°C were shifted to 41°C; at 15 min (a) or 60 min (b) after the temperature shift, cells were collected and ruptured, and their cytoplasmic contents were separated by centrifugation as described in Materials and Methods. Fractions were collected, their A_{260} was determined, and aliquots of each were separated by one-dimensional SDS-PAGE, electroblotted, immunoreacted with anti-p22 antiserum, and stained (inserts). The direction of sedimentation is from right to left. The regions of the gradient containing polysomes (p) and monosomes (m) are indicated.

cross-linking coupled with immunocytochemical localization.

Our electrophoretic and immunological data indicate that p22 does not undergo a detectable posttranslational modification during heat shock, which might have explained its increased affinity for the ribosome (or its decreased affinity for something else or both). Some other alteration(s) must be involved. One possible explanation for the increased ribosomal affinity of p22 is that ribosomes in heat-shocked cells undergo a change in conformational state, resulting in the generation of a strong binding site for p22. An overall shift in ribosome conformation could cause changes in the affinities of a number of ribosome-associated proteins. This could explain the other changes seen in the array of proteins found on ribosomes isolated from heat-shocked cells (Fig. 2).

We have some indirect evidence that a change in ribosome conformation occurs and is correlated with an increased affinity for p22. First, the ribosomes of long-starved cells (>15 h in a low-ionic-strength salt solution) undergo a change in conformation which manifests itself in a number of ways, one of which is an altered affinity for a number of antibiotics (15). In these same cells, p22 becomes strongly associated with ribosomes (data not shown). Second, heat shock also induces a decreased sensitivity to the inhibitory effects of both cycloheximide and emetine (unpublished data), and because these antibiotics interact with different sites on the two ribosomal subunits (36), this must occur because of some overall change in ribosome conformation. Finally, when growing cells are transferred to certain starvation media and heat shocked, rapid and selective degradation of the ribosomes of the cells occurs (13). However, cells can be preconditioned to survive this heat shock and not degrade their ribosomes by a variety of treatments, all of which coincidentally induce the strong association of p22 with the ribosome (these include starvation for periods of greater than 15 h, allowing cells to enter a plateau state of growth, and heat shock of growing cells). A conformational change in the ribosome induced by the various pretreatments may play a role in stabilizing the ribosome so that during the heat shock in starvation media it is not susceptible to the increased degradative activity.

As to the functional significance of these observations, the kinetics of appearance and disappearance of a tightly bound p22 correlate with a changing pattern of protein synthesis which occurs during heat shock. These include the resumption of non-hsp synthesis and the decrease in hsp synthesis while cells are still at the hsp-inducing temperature. Our preliminary evidence indicates that both of these changes in protein synthesis pattern are regulated in part at the level of translation. Because starved cells having a tightly associated p22 show a normal hsp synthesis pattern when shifted to 41°C but show a lesser degree of suppression of non-hsp synthesis (unpublished data), the presence of p22 appears to be more likely associated with non-hsp mRNA usage than with hsp mRNA nonusage. Whether the presence of p22 plays a direct role in the transition of hsp synthesis to non-hsp synthesis during heat shock or simply monitors this change remains to be proven. A combination of genetic and in vitro analyses will be required to unequivocally determine this.

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LITERATURE CITED

1. **Allis, C. D., R. L. Allen, J. C. Wiggens, L. G. Chicoine, and R. Richman.** 1984. Proteolytic processing of H1-like histones in chromatin: a physiologically and developmentally regulated event in *Tetrahymena* micronuclei. J. Cell Biol. **99**:1669–1677.
2. **Ballinger, D. G., and M. L. Pardue.** 1983. The control of protein synthesis during heat shock in *Drosophila* cells involves altered polypeptide elongation rates. Cell **33**:103–114.
3. **Banerji, S. S., N. G. Theodorakis, and R. I. Morimoto.** 1984. Heat shock-induced translational control of HSP70 and globin synthesis in chicken reticulocytes. Mol. Cell. Biol. **4**:2437–2448.
4. **Bienz, M., and J. B. Gurdon.** 1982. The heat shock response in *Xenopus* oocytes is controlled at the translational level. Cell **29**:811–819.
5. **Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf.** 1970. Methods in immunology, 2nd ed., p. 28–30 and 181–191. W. A. Benjamin, Inc., New York.
6. **Duncan, R., and J. W. Hershey.** 1984. Heat shock-induced translational alterations in HeLa cells. Initiation factor modifications and the inhibition of translation. J. Biol. Chem. **259**:11882–11889.
7. **Fink, K., and E. Zeuthen.** 1980. Heat shock proteins in *Tetrahymena* studied under growth conditions. Exp. Cell Res. **128**:23–30.
8. **Glover, C. V. C.** 1982. Heat shock induces rapid dephosphorylation of a ribosomal protein in *Drosophila*. Proc. Natl. Acad. Sci. USA **79**:1781–1785.
9. **Gorenstein, C., and J. R. Warner.** 1976. Coordinate regulation of the synthesis of eukaryotic ribosomal proteins. Proc. Natl. Acad. Sci. USA **73**:1547–1551.
10. **Gorovsky, M. A., C. V. C. Glover, S. D. Guttmann, K. J. Vavra, S. Horowitz, and D. S. Pederson.** 1982. Stress-induced changes in nuclear proteins of *Tetrahymena*, p. 299–308. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Guttman, S. D., C. V. C. Glover, C. D. Allis, and M. A. Gorovsky.** 1980. Heat shock, deciliation, and release from anoxia induce the synthesis of the same set of polypeptides in starved *T. pyriformis*. Cell **22**:299–307.
12. **Hallberg, R. L., and P. J. Bruns.** 1976. Ribosome biosynthesis in *Tetrahymena pyriformis*: regulation in response to nutritional changes. J. Cell Biol. **71**:383–394.
13. **Hallberg, R. L., K. W. Kraus, and R. C. Findly.** 1984. Starved *Tetrahymena thermophila* cells that are unable to mount an effective heat shock response selectively degrade their rRNA. Mol. Cell. Biol. **4**:2170–2179.
14. **Hallberg, R. L., K. W. Kraus, and E. M. Hallberg.** 1985. Induction of acquired thermotolerance in *Tetrahymena thermophila*: effects of protein synthesis inhibitors. Mol. Cell. Biol. **5**:2061–2069.
15. **Hallberg, R. L., P. G. Wilson, and C. A. Sutton.** 1981. Regulation of ribosome phosphorylation and antibiotic sensitivity in *Tetrahymena thermophila*: a correlation. Cell **26**:47–56.
16. **Hickey, E. D., and L. A. Weber.** 1982. Modulation of heat shock polypeptide synthesis in HeLa cells during hyperthermia and recovery. Biochemistry **21**:1513–1521.
17. **Hickey, E. D., and L. A. Weber.** 1982. Preferential translation of heat-shock mRNAs in HeLa cells, p. 199–206. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Kennedy, I. M., R. H. Burdon, and D. P. Leader.** 1984. Heat shock causes diverse changes in the phosphorylation of the ribosomal proteins of mammalian cells. FEBS Lett. **169**: 267–273.
19. **Kruger, C., and B. J. Benecke.** 1981. In vitro translation of *Drosophila* heat-shock and non-heat-shock mRNAs in heterologous and homologous cell-free systems. Cell **23**:595–603.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lasky, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:93–104.
22. **Lastick, S. M., and E. H. McConkey.** 1976. Exchange and stability of HeLa ribosomal proteins *in vivo*. J. Biol. Chem. **251**:2867–2875.
23. **Lindquist, S.** 1981. Regulation of protein synthesis during heat shock. Nature (London) **293**:311–314.
24. **McAlister, L., S. Stausberg, A. Kulaga, and D. B. Finkelstein.** 1979. Altered patterns of protein synthesis induced by heat shock of yeast. Curr. Genet. **1**:63–74.
25. **Miller, M. J., N. H. Xuong, and E. P. Geiduschek.** 1979. A response of protein synthesis to temperature shift in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **76**:5222–5225.
26. **Olsen, A. S., D. F. Triemer, and M. M. Sanders.** 1983. Dephosphorylation of S6 and expression of the heat shock response in *Drosophila melanogaster*. Mol. Cell. Biol. **3**:2017–2027.
27. **Petridou, B., M. Cuny, M. F. Guerin, and F. Hayes.** 1983. Ribosomal subunits and ribosomal proteins of *Tetrahymena thermophila*. Effect of the presence of iodoacetamide during ribosome extraction on the properties of the subunits. Eur. J. Biochem. **135**:424–434.
28. **Scharf, K. D., and L. Nover.** 1982. Heat shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. Cell **30**:427–437.
29. **Schlesinger, M. J., M. Ashburner, and A. Tissieres (ed.).** 1982. Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Scott, M. P., and M. L. Pardue.** 1981. Translational control in lysates of *Drosophila melanogaster* cells. Proc. Natl. Acad. Sci. USA **78**:3353–3357.
31. **Storti, R. V., M. P. Scott, A. Rich, and M. L. Pardue.** 1980. Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. Cell **22**:825–834.
32. **Sutton, C. A., M. Ares, and R. L. Hallberg.** 1978. Cycloheximide resistance can be mediated through either ribosomal subunit. Proc. Natl. Acad. Sci. USA **75**:3158–3162.
33. **Thomas, G. P., and M. B. Mathews.** 1982. Control of polypeptide chain elongation in the stress response: a novel translational control, p. 207–213. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
35. **Vaitukaitis, J. L.** 1981. Production of antisera with small doses of immunogen: multiple intradermal injections. Methods Enzymol. **73**:47–52.
36. **Vazquez, D.** 1979. Inhibitors of protein biosynthesis. Springer-Verlag, New York.
37. **Wilhelm, J. M., P. Spear, and C. Sax.** 1982. Heat shock proteins in the protozoan *Tetrahymena*: induction by protein synthesis inhibition and possible role in carbohydrate metabolism, p. 309–314. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.