

Durable Synthesis of High Molecular Weight Heat Shock Proteins in G₀ Cells of the Yeast and Other Eucaryotes

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ABSTRACT We report that eucaryotic cells were induced to synthesize a specific class of heat shock proteins (hsps) when they entered the resting state, G₀. This finding was originally made with *Saccharomyces cerevisiae* cells by taking advantage of the system in which we can distinguish between G₁ arrests leading to G₀ and those that do not result in G₀ (Iida, H., and I. Yahara, 1984, *J. Cell Biol.* 98:1185–1193). Similar observations were subsequently made with higher eucaryotic cells including chick embryonic fibroblasts (CEF), mouse T lymphocytes, and *Drosophila* GM1 cells. The induction of hsps in G₀ cells was distinct from that in heat-shocked cells in two respects. First, hsps with molecular weight around 25,000 were not induced in G₀ cells, whereas most, if not all, high molecular weight (HMW) hsps were commonly induced both in G₀ cells and in heat-shocked cells. Second, in contrast to the transient synthesis of hsps in heat-shocked cells, G₀ cells continued to synthesize hsps at the stimulated rate for a relatively long period. These results suggest the possibility that high molecular weight hsps might function in a transition from the proliferating state to G₀ or in maintaining G₀ in the eucaryote.

The transition from the proliferating state to the resting state, G₀, has been revealed with experimental systems *in vitro* to have the same features as that observed *in vivo* (2, 18). Referring to cellular events and characteristics specifically associated with the progress toward G₀ in fibroblasts and lymphocytes, we have found with yeast cells that blocking the process of the cell cycle at specific point(s) in G₁ resulted in the G₀ state (11). Growth arrest produced by sulfur starvation or temperature sensitive mutation in *CDC25*, *CDC33*, *CDC35*, or *ILS1* gene lead yeast cells to enter G₀ while arrest yielded by α factor or temperature sensitive mutation in *CDC4*, *CDC24*, or *CDC28* gene do not.

Using this system of the yeast, we have made an attempt to identify proteins specifically or preferentially synthesized during the transition from G₁ to G₀ or in G₀ in the hope that these proteins might function as a negative-regulatory machinery in eucaryotes. We report that at least nine proteins were synthesized specifically in G₀ yeast cells and six of them were identified with those known as heat shock proteins (hsps) (for review, see references 1 and 37). Stimulation of the synthesis of hsps in G₀ was found also with higher organisms, suggesting that this phenomenon would be common to all the

eucaryotes. The synthesis of hsps has been known to be transiently induced under various restrictive conditions for growth besides heat shock (1). Based on the present and previous observations, we suggest that hsps might be involved in a cellular machinery that directs cells toward G₀.

MATERIALS AND METHODS

Yeast Strains: Genotypes and sources of strains were tabulated in the previous paper (11).

Media: Media used for cultivation of yeast cells were described elsewhere (11). Minimum essential medium (Nissui, Tokyo, Japan), RPMI 1640 medium (Gibco Laboratories, Inc., Grand Island, NY) and modified Schneider's medium (MSM) (30) were used for cultures of chick embryonic fibroblasts (CEF), mouse lymphocytes, and *Drosophila* GM1 cells, respectively. Fetal bovine serum (FBS) (Gibco Laboratories) was supplemented when indicated.

Cell Culture and Labeling of Proteins: Cells of *cdc* mutants and their parental strain (A364A) were grown with shaking at 23°C in low-methionine medium, a modified SYE medium (11) in which methionine was omitted from yeast nitrogen base (Difco Laboratories, Inc., Detroit, MI) and the

Abbreviations used in this paper: CEF, chick embryo fibroblasts; FBS, fetal bovine serum; hsp, heat shock protein; HMW, high molecular weight; LMW, low molecular weight; MSM, modified Schneider's medium.

concentration of yeast extract was reduced to 0.025%. The incorporation of [³⁵S]methionine increased up to eightfold in this low methionine medium without affecting the growth of yeast cells. X2180-1A cells were grown at 30°C in SD10 and starved for sulfur in SD10-S medium. For labeling of *cdc* mutants and A364A, exponentially growing cells with the initial concentration of 5×10^5 cells/ml or cells arrested at nonpermissive temperature (38°C for *cdc28* and 36°C for others) for indicated periods were pulse-labeled with 10–50 μ Ci/ml L-[³⁵S]methionine (Amersham Corp., Tokyo, Japan) with the final specific activity of 0.26–13 Ci/mmol for 10 min, and chased for an additional 3 min by addition of nonradioactive methionine to 5 mg/ml. The initial cell density of A364A should be 5×10^5 /ml or lower upon the temperature shift because otherwise the culture of this strain could almost reach the stationary phase 6 h after the shift to 36°C. For labeling of sulfur-starved X2180-1A cells, cells grown in SD10 medium (1×10^7 cells/ml) were incubated in SD10-S medium up to 40 h and were pulse-labeled with 10 μ Ci/ml [³⁵S]methionine (5.3 Ci/mmol) for 10 min at 30°C and chased for 3 min. The radiolabeled cells were chilled, washed twice with 20 mM Tris HCl (pH 8.8), 2 mM CaCl₂ (Tris/CaCl₂), and kept frozen at –80°C until use.

GM1, a *D. melanogaster* cell line of embryonic origin (31) was provided by T. Miyake (Mitsubishi-Kasei Institute of Life Science, Tokyo, Japan). GM1 cells were grown at 25°C in a spinner flask containing MSM supplemented with 10% FBS (30). Exponentially growing cells (4×10^5 cells/ml) and those of the stationary phase (2×10^6 cells/ml) were separately washed with methionine and Bacto-peptone-free MSM-10% FBS, resuspended in the same medium, and incubated at 25°C for 15 min. Each culture was then divided into two equal parts, after which one was incubated at 25°C and the other at 37°C for 15 min in plastic tubes. They were finally pulse-labeled for 30 min with [³⁵S]methionine (1,200 Ci/mmol, 0.1 mCi/ml), washed with chilled MSM, and frozen at –80°C.

Secondary cultures of CEF were grown in minimum essential medium containing 5% FBS at the initial cell density of 2×10^4 /cm² in tissue culture flasks. 1.5- and 7-d cultures were used as an exponentially growing culture and as a stationary culture, respectively. These cultures received 10 μ Ci/ml of [³⁵S]methionine (the final specific activity, 0.1 Ci/mmol) and 20 mM HEPES, pH 7.4, and were incubated for 1 h at 37°C for 45°C. The radiolabeled cells were washed twice with PBS and frozen at –80°C.

Methods for the preparation of resting and growing mouse lymphocytes used in this study were previously described in detail (18). G₀ T lymphocytes were purified from spleen cells of DBA/2 mice by Ficoll-Paque centrifugation and passage through Nylon wool columns. Day 2 and day 4 cultures of stimulated lymphocytes with Con A were used as the sources of growing and G₁ lymphocytes, respectively. Lymphocytes to be labeled were washed with methionine-free RPMI 1640 medium containing 5% FBS and suspended in the same medium at a cell density of 3.3×10^6 /ml. The cell suspensions received 0.1 mCi/ml [³⁵S]methionine (1,200 Ci/mmol) and were incubated for 1 h at 37°, 42°, or 45°C, after which the cells were washed and frozen at –80°C.

Protein Extraction and Two-dimensional Gel Electrophoresis: Methods for protein extraction from whole cells are essentially the same as those described by Miller et al. (27) but with slight modifications. The extraction was performed at 0–5°C. Briefly, frozen yeast cells were vortexed for 4×30 s with 0.3 g of 0.5 mm-glass beads and 2.5 μ l of 100 mM phenylmethylsulfonyl fluoride (PMSF). The lysates received 0.2 ml Tris/CaCl₂ and 10 μ l of 1 mg/ml Micrococcal nuclease (Worthington Biochemical Corp., Freehold, NJ), and were incubated for 5 min, after which they were mixed with 20 μ l of 2% SDS-10% 2-mercaptoethanol and 20 μ l of 1 mg/ml pancreatic DNase I (Worthington Biochemical Corp., Code:DPFF), 2 mg/ml RNase A (Worthington Biochemical Corp., Code:RAF) made up to 0.5 M Tris-HCl (pH 7.0), 50 mM MgCl₂. The mixtures were then lyophilized and dissolved in lysis buffer of O'Farrell (32) at room temperature.

CEF, mouse lymphocytes, and GM1 cells were sonicated in 212.5 μ l of Tris/CaCl₂ containing 1.2 mM phenylmethylsulfonyl fluoride and 47 μ g/ml Micrococcal nuclease. Cell extracts were prepared in the same way as for yeast cells.

Two-dimensional polyacrylamide gel electrophoresis (NEPHGE/SDS PAGE) (33) was used for analysis of proteins in whole cell extracts. 20- μ l aliquots of the cell extracts were electrophoresed in the first dimension at 400 V for 4 h in 2.5 \times 130-mm glass tubes containing 4% acrylamide/bisacrylamide gel, 9.2 M urea, 2% Nonidet P-40, and 2% Ampholine (pH 3.5–10, LKB, Bromma, Sweden). Electrophoresis in the second dimension was carried out with 11% polyacrylamide gel containing SDS. The gels were stained with Coomassie Brilliant Blue, after which fluorograms were made up with EN³HANCE (NEN, Boston, MA). The dried gels were exposed to Kodak X-Omat S film (XS-1) together with standard spots containing a series of known ³⁵S radioactivities.

Densitometry of Two-dimensional Gels: Spots on XS-films were analyzed and quantitated with the interactive pattern analysis system (15) which was composed of a computer (PDP 11/70, Digital Equipment Co.,

Maynard, MA) coupled with a drum scan densitometer (Model 2604, Abe Sekkei Co., Tokyo, Japan) and an interactive image display (NAC Inc., Tokyo, Japan). The optical density of every 0.25 mm² in fluorographic films was measured and converted into the radioactivity according to the standard curve of optical density versus radioactivity obtained on the same films. Radioactivities contained in the spots in question and those in the whole gels were calculated. Methods for this two-dimensional densitometric analysis will be described in detail (Minamikawa, R., N. Hata, and T. Kaminuma, manuscript in preparation).

RESULTS

Identification of Yeast G₀ Proteins

To investigate G₀ proteins of yeast cells, we have used three *cdc* mutants (*cdc25*, *cdc33*, and *cdc35*) and sulfur starvation, which led to G₀ arrest of these cells (11). The results were compared with those obtained with other *cdc* mutant strains such as *cdc28*, which was not arrested in G₀ at nonpermissive temperature. Since the method for growth arrest of *cdc* mutants was shift-up of the temperature, hsp's could also be induced by the shift-up regardless of growth arrest. In addition, hsp's may be induced also by sulfur starvation because hsp's are known to be induced under stressful conditions (1, 8, 13, 16, 19–22) and sulfur starvation is possibly stress. To discriminate between possibly simultaneously inducible G₀ proteins and hsp's, we used two criteria. First, synthesis of hsp's solely induced by heat shock or by stress (13, 19, 21) continues only for a short period up to 1 h and ceases thereafter, perhaps due to feedback regulation of mRNA synthesis by hsp's (27, and see below). Second hsp's but not G₀ proteins are transiently induced at nonpermissive temperature in the parental strain A364A and other *cdc* mutants (e. g., *cdc28*) that were arrested but did not enter G₀ (11).

Exponentially growing cells at 23°C of three *cdc* mutants, *cdc25*, *cdc33*, and *cdc35*, and the parental strain A364A were incubated at 36°C. The cells were pulse-labeled with [³⁵S]methionine for 10 min, at 1 and 6 h after the temperature shift. The proteins synthesized during the pulse were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-NEPHGE/SDS PAGE) (33).

We found with *cdc25* cells that 36 proteins were continuously synthesized at 36°C up to 6 h after the shift of temperature while they were synthesized only slowly, if at all, at 23°C (Fig. 1, A–C). For instance, a protein with molecular weight of 73,000 (p73; indicated by an arrow in Fig. 1C) was not synthesized in growing *cdc25* cells at 23°C. Synthesis of this protein was induced by transfer to 36°C and remained stimulated even 6 h later (Fig. 1 and Table I). P73 was also synthesized in sulfur-starved cells and in cells of *cdc33* and *cdc35* mutants that had been incubated at 36°C for 6 h, although the rate of the synthesis was relatively low compared with *cdc25* cells at 36°C (Fig. 2A and Table I). Synthesis of p73 was not significantly detected in *cdc28* cells arrested at 38°C (Fig. 2, B and C and Table I) or in *cdc25* cells arrested by α factor at 23°C (data not shown). These results showed that p73 satisfied criteria for G₀ protein.

By contrast, a protein with molecular weight of 55,000 (Fig. 1) was synthesized in *cdc25* or *cdc35* cells at 36°C, but was not synthesized in *cdc33* cells at the same temperature (Table I). Accordingly, p55 did not meet the criteria for G₀ protein. Based upon quantitative data shown in Table I, nine out of the 36 proteins were commonly observed to be durably synthesized under restrictive conditions by sulfur starvation or with *cdc35* or *cdc33* and, therefore, were designated as putative G₀ proteins of the yeast. They are proteins with molecular

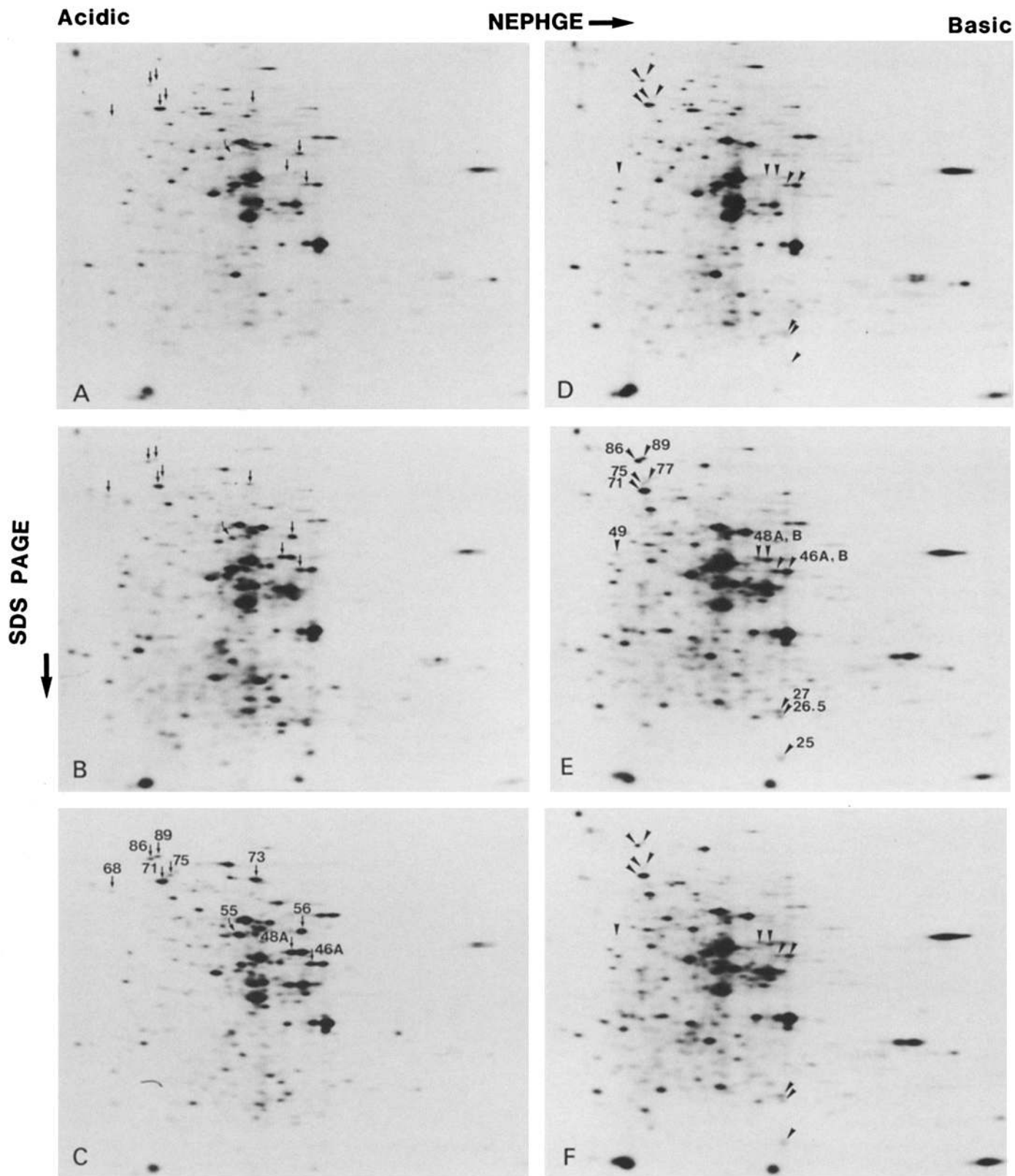


FIGURE 1 2D-NEPHGE/SDS PAGE analysis of [^{35}S]methionine-labeled yeast proteins synthesized in *cdc25* cells and A364A cells at 23° and 36°C. Exponentially growing cells at 23°C and cells shifted to 36°C were pulse-labeled with [^{35}S]methionine (10 $\mu\text{Ci/ml}$, 0.26 Ci/mmol) for 10 min and chased for 3 min with nonradioactive L-methionine (5 mg/ml). Radioactivity of 4×10^4 dpm was analyzed for each sample. (A) *cdc25* cells grown and pulsed at 23°C; (B and C) *cdc25* cells pulsed 1 and 6 h after the shift from 23° to 36°C, respectively. (D) Cells of A364A, the parental strain of the *cdc25* mutant, grown, and pulsed at 23°C. (E and F) A364A cells pulsed 1 and 6 h after the shift, respectively. Arrows and arrowheads indicate G_0 -induced proteins and hsp, respectively. A bent arrow indicates a protein, p55, whose synthesis is induced in arrested *cdc25* or *cdc35* cells but not in arrested *cdc33* cells or in sulfur-starved cells (see the text). Numerals affixed to arrows or arrowheads indicate molecular weights of proteins in kilodaltons.

TABLE I

Synthesis of Individual Proteins in Permissive and Nonpermissive Conditions for Growth

| Induction of arrest | Incubation condition | Total protein synthesis* cpm/10 min/ μ g | Relative rate of individual protein synthesis [†] | | | | | | | | | | | |
|----------------------------|----------------------|---|--|---------------------|---------------------|----------------|---------------------|----------------|----------------|------|---------------------|------|---------------------|-----|
| | | | p89 | p86 | p75 | p73 | p71 | p68 | p56 | p55 | p48A | p48B | p46A | p27 |
| <i>cdc25</i> | 23°C | 1,256 | 1.1 | 12 | 1.1 | 4.9 | 250 | 1.6 | 93 | 6.0 | 7.7 | 21 | 60 | 31 |
| | 36°C, 1 h | 2,321 | 28 | 39 | 18 | 69 | 334 | 24 | 202 | 131 | 191 | 389 | 157 | 123 |
| | 36°C, 6 h | 809 | 94 | 129 | 50 | 1373 | 864 | 34 | 1194 | 1491 | 1691 | 3203 | 548 | 17 |
| <i>cdc33</i> | 23°C | 563 | 1.1 | 13 | 1.6 | 1.1 | 38 | 2.9 | 4.8 | 15 | 3.7 | 34 | 10 | 41 |
| | 36°C, 1 h | 545 | 19 | 18 | 4.8 | 2.8 | 183 | 13 | 66 | 16 | 14 | 81 | 58 | 142 |
| | 36°C, 6 h | 383 | 37 | 75 | 6.7 | 9.1 | 351 | 18 | 75 | 10 | 15 | 100 | 78 | 76 |
| <i>cdc35</i> | 23°C | 1,333 | 1.4 | 28 | 0.9 | 29 | 151 | 7.2 | 74 | 17 | 37 | 55 | 63 | 28 |
| | 36°C, 1 h | 1,894 | 49 | 42 | 12 | 67 | 182 | 31 | 567 | 66 | 317 | 676 | 124 | 56 |
| | 36°C, 6 h | 416 | 171 | 209 | 373 | 479 | 1150 | 54 | 820 | 1084 | 338 | 1170 | 209 | 6.3 |
| Sulfur starvation | 0 h | 110,045 | 1.2 | 27 | 1.2 | 1.2 | 296 | 1.2 | 8.9 | 4.8 | 18 | 67 | 73 | 24 |
| | 1 h | 51,524 | 38 | 78 | 15 | 10 | 746 | 53 | 46 | 4.6 | 238 | 390 | 157 | 47 |
| | 40 h | 15,779 | 56 | 96 | 136 | 67 | 3293 | 59 | 157 | 3.2 | 541 | 2851 | 194 | 15 |
| <i>cdc28</i> | 23°C | 1,651 | 1.4 | 39 | 1.0 | 1.4 | 240 | 1.4 | 27 | 3.9 | 3.9 | 42 | 49 | 24 |
| | 38°C, 1 h | 3,323 | 69 | 87 | 25 | 1.9 | 510 | 1.4 | 80 | 6.0 | 114 | 258 | 270 | 56 |
| | 38°C, 6 h | 1,625 | 18 | 19 | 5.5 | 1.0 | 311 | 1.0 | 32 | 4.0 | 31 | 742 | 33 | 55 |
| A364A | 23°C | 1,092 | 0.6 | 34 | 1.7 | 1.7 | 263 | 1.7 | 12 | 4.6 | 2.3 | 9.1 | 62 | 30 |
| | 36°C, 1 h | 2,719 | 62 | 153 | 14 | 1.9 | 502 | 4.7 | 15 | 9.3 | 28 | 156 | 110 | 90 |
| | 36°C, 6 h | 4,608 | 15 | 65 | 8.5 | 0.5 | 375 | 1.5 | 6.0 | 7.5 | 6.5 | 72 | 40 | 37 |
| Identification of proteins | | | G ₀ /hsp | G ₀ /hsp | G ₀ /hsp | G ₀ | G ₀ /hsp | G ₀ | G ₀ | — | G ₀ /hsp | hsp | G ₀ /hsp | hsp |

* Cells were pulse-labeled with [³⁵S]methionine under the conditions described in the text. The specific activities of [³⁵S]methionine used for labeling were 0.3 Ci/mmol (20 μ Ci/ml) for A364A and *cdc* mutants, and 10 Ci/mmol (20 μ Ci/ml), for X2180-1A. Method for determination of radioactivity associated with trichloroacetic acid-insoluble materials and method for determination of protein content were previously described (11). Values listed in this column indicate radioactivity (cpm) incorporated into proteins per microgram protein during 10-min pulse.

[†] The synthesizing rate of individual proteins was obtained by densitometry of two-dimensional gels followed by conversion into radioactivity (see Materials and Methods). Each value listed above was then calculated by dividing the synthesizing rate of individual proteins by the synthesizing rate of the total proteins followed by multiplying by 10⁶. The background level given by densitometry was $1-2 \times 10^{-6}$ but was not subtracted from the above values.

weights of 89,000 (p89), 86,000 (p86), 75,000 (p75), 73,000 (p73), 71,000 (p71), 68,000 (p68), 56,000 (p56), 48,000 (p48A), and 46,000 (p46A) (Table I).

The synthesis of four G₀ proteins, p86, p71, p56, and p46A, was significantly detected even in exponentially growing cells of *cdc25*, and was stimulated to a large extent after the shift of temperature (Fig. 1 and Table I). The background synthesis of the others p89, p75, p73, p68, and p48A, was negligible in the same strain at 23°C.

P48A (the acidic form of p48) and p48B (the basic form of p48) are composed of similar polypeptides (unpublished observations). Criteria for G₀ proteins used in the present study are met with p48A but not with p48B (Table I). P46A and p46B are similar but not identical in peptide maps (unpublished observations). P46A is a G₀ protein but p46B is not.

Relationship of G₀ Proteins to Heat Shock Proteins

To identify hsp of yeast cells, A364A cells grown at 23°C were exposed to 36°C and pulse-labeled with [³⁵S]methionine 1 and 6 h after the temperature shift (Fig. 1, D-F). We detected thirteen hsp listed in Table II. These results are consistent in part with those of others (25, 27). We noted that the relative synthesizing rate of the thirteen hsp in A364A cells was higher 1 h after the shift than 6 h after (Fig. 1, D-F and Table I).

We unexpectedly found that six out of the nine G₀ proteins were hsp. Conversely, six out of the ten high molecular weight (HMW) hsp, those with molecular weight over 45,000, were G₀ proteins, whereas none of the low molecular weight (LMW) hsp, those with molecular weight around 27,000, was a G₀ protein (Table II). Evidence that these six G₀ proteins are identical to hsp was further provided by limited proteolysis of these proteins extracted from spots on two-dimensional gels. An example is shown in Fig. 3, A and B, which indicates that p89 induced in G₀-arrested *cdc25* cells and p89 induced

by heat shock in A364A cells are identical.

Proteins that are simultaneously G₀ proteins and hsp are distinguishable from simple hsp by the criteria described above. The rate of synthesis of hsp89, hsp75, and hsp48A increased in *cdc25*, for example, after the shift-up of temperature at least up to 6 h, whereas that of a simple hsp, hsp27, was accelerated in the same strain 1 h after the shift-up but relatively decreased 6 h after. It should be noted that G₀ and heat shock proteins behaved like simple hsp in *cdc28* and A364A, which did not rest in G₀ at nonpermissive temperature (Table I).

As described above, four (p86, p71, p56, p46A) out of nine G₀ proteins are synthesized also in the proliferating state although the relative rate of their synthesis in growing cells is lower than that in G₀ cells. The question may be raised as to whether the proteins identified as G₀ proteins were not induced in G₀ but the synthesis of them merely persisted even in G₀ where the overall protein synthesis fell. The synthesizing rates in G₀-arrested *cdc25* cells of p86, p56, and p46A per microgram total proteins were estimated to be 6.9, 8.3, and 5.9 times higher than those in the growing state, respectively (Table I). This suggests that the synthesis of these three proteins was really induced or accelerated in G₀. However, the rate per microgram total proteins of p71 in G₀ yeast cells was comparable with that in growing cells. Thus an increase in the relative synthesizing rate of p71 to that of the total proteins in G₀ may be accounted for by persisting synthesis of this protein in G₀ rather than induction.

Stimulation of Synthesis of HMW-hsp in G₀ of Higher Eucaryotes

In accord with the report by Kelley and Schlesinger (16, 17), chick cells responded to heat shock and synthesized four HMW-hsp, hsp89, hsp75, hsp70A, and hsp70B. In two-dimensional gels, yeast hsp89 and hsp75 appeared to resemble chick hsp89 and hsp75, respectively (Fig. 4). Yeast hsp89 and

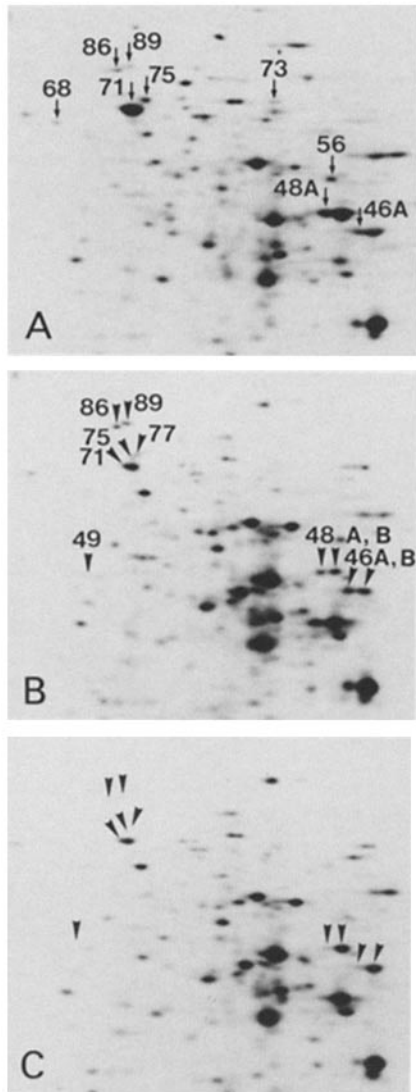


FIGURE 2 2D-NEPHGE/SDS PAGE analysis of [³⁵S]methionine-labeled yeast proteins synthesized in sulfur-starved cells and *cdc28* cells. (A) X2180-1A cells starved for sulfur for 40 h were pulsed with [³⁵S]methionine. (B and C) *cdc28* cells were pulsed after incubation at 38°C for 1 and 6 h, respectively. Arrows and arrowheads indicate G₀-proteins and hsp, respectively.

hsp75 were indistinguishable from the corresponding chick hsp in molecular weight and more acidic than the chick hsp. Both yeast hsp89 and the corresponding chick hsp showed trails in NEPHGE.

If HMW-hsp function in transition to G₀ or maintenance of G₀, they would be synthesized also in G₀ cells of other organisms. We have examined CEF, mouse lymphocytes, and cultured *Drosophila* cells in this respect.

Exponentially growing cells (1.5 × 10⁶/25 cm² flask) and quiescent cells of confluent culture (4 × 10⁶/flask) of CEF were separately labeled with [³⁵S]methionine for 1 h. The cells were heat-shocked before pulse-labeling if indicated. Fixed radioactivities (4 × 10⁴ dpm) of the total cell extracts were analyzed in 2D-NEPHGE/SDS PAGE. The relative synthesizing rate of HMW-hsp including hsp89, hsp75, hsp70A, and hsp70B was found greater in a confluent culture than in an exponentially growing culture (Fig. 5, A and B). The synthesis of LMW-hsp such as hsp26 was not stimulated in resting

CEF cells (data not shown). The extent of the stimulation by transition to G₀ varied with different hsp and was not correlated to that of the synthesis induced by heat shock (Fig. 5 C). For instance, the synthesis of hsp70B was stimulated to a higher degree in heat-shocked cells than in G₀ cells, whereas that of hsp75 was stimulated to the same degree in both cultures. A further increase in the synthesizing rate of the four HMW-hsp was observed when a confluent CEF culture was heat-shocked (data not shown). A limited proteolysis study showed that hsp synthesized in confluent culture are identical to those induced in heat-shocked cells. Examples for hsp70A and hsp70B are presented in Fig. 3, C–F.

Resting and growing cells of mouse T lymphocytes were prepared as described (18). The synthesis of hsp89, hsp75,

TABLE II
G₀ Proteins and Hsp in Yeast Cells

| G ₀ protein | Heat shock protein |
|------------------------|--------------------|
| p89 | hsp89 |
| p86 | hsp86 |
| — | hsp77 |
| p75 | hsp75 |
| p73 | — |
| p71 | hsp71 |
| p68 | — |
| p56 | — |
| — | hsp49 |
| p48A | hsp48A and B |
| p46A | hsp46A and B |
| — | hsp27 |
| — | hsp26.5 |
| — | hsp25 |

Proteins aligned in the same horizontal lines are those that are simultaneously G₀ and heat-shock proteins.

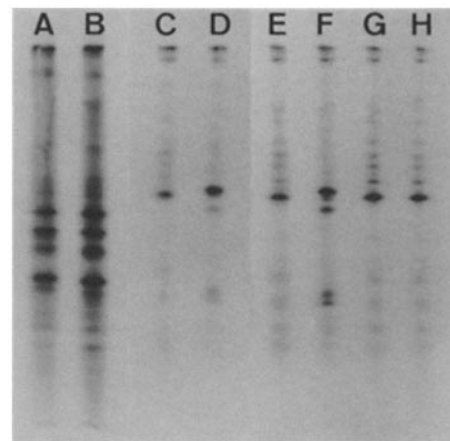


FIGURE 3 Peptide mapping by limited proteolysis of G₀ proteins and hsp. [³⁵S]methionine-labeled proteins extracted from cells were separated by 2D-NEPHGE/SDS PAGE. The spots to be examined were cut out from the gels and subjected to proteolysis with *Staphylococcus aureus* V8 protease (5 ng/each slot) according to the methods described by Cleveland et al. (5). Digested products were detected by fluorography. (A) hsp89 of G₀-arrested *cdc25* cells that were incubated at 36°C for 6 h; (B) hsp89 of A364A cells heat-shocked at 36°C for 1 h; (C) hsp70A of confluent CEF; (D) hsp70B of confluent CEF; (E) hsp70A of growing CEF that were heated at 45°C for 1 h; (F) hsp70B of growing CEF that were heated at 45°C for 1 h; (G) hsp70A of resting mouse T lymphocytes; (H) hsp70A of growing mouse T lymphocytes.

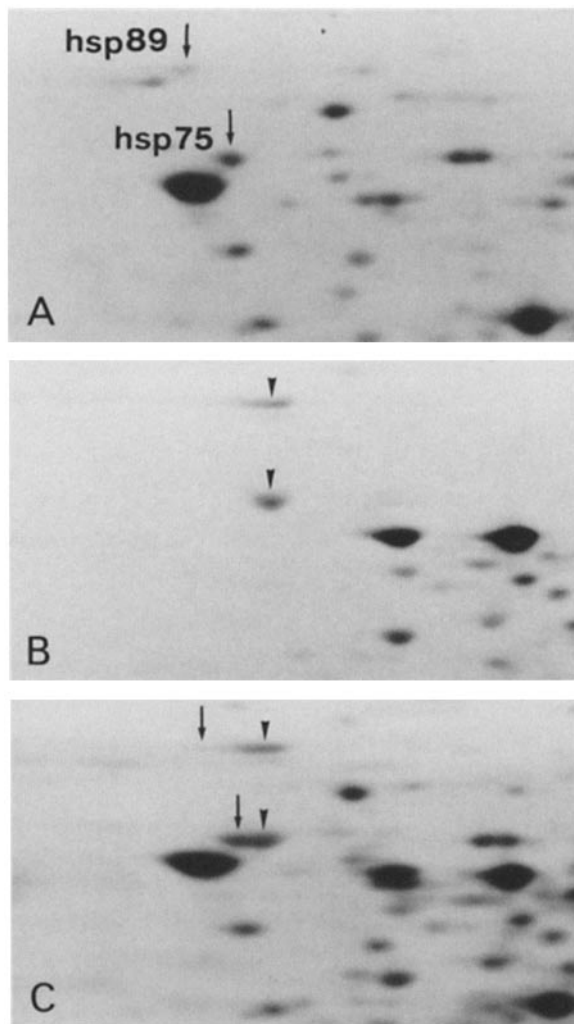


FIGURE 4 Co-electrophoresis of yeast and CEF proteins in 2D-NEPHGE/SDS PAGE. (A) Yeast proteins (4×10^4 dpm) synthesized in sulfur-starved X2180-1A cells; (B) CEF proteins (4×10^4 dpm) synthesized in heat-shocked CEF; (C) A mixture of samples used in A and B. Arrows indicate yeast hsp89 and hsp75. Arrowheads indicate CEF hsp89 and hsp75.

and hsp70 was found to be stimulated in G_0 T cells compared with growing T cells (Fig. 6). hsp70B was not detected in mouse lymphocytes at all. It would be pertinent to note that the synthesis of these three hsps was not significantly stimulated by heat shock at 42°C or 45°C (data not shown). Nevertheless, peptide mapping indicate that these proteins are similar to the corresponding hsps of CEF. For instance, hsp70A synthesized in resting or growing mouse T lymphocytes is similar in peptide composition to hsp70A of the chicken which is stimulatory by heat shock (Fig. 3). A G_0 -specific protein with molecular weight of 68,000 was detected in both chick and mouse cells (Figs. 5 and 6) but was not an hsp of these species (data not shown).

Since the heat shock response has been most extensively analyzed in *Drosophila* (1), we have tested whether the synthesis of hsps was stimulated in G_0 *Drosophila* cells. A cell line, GM1, originated from an embryo of *D. melanogaster* was used in this study. Cultured cells at the exponentially growing phase and the stationary phase were analyzed by the method described above. When indicated, cells grown at 25°C were heat-shocked at 36°C for 15 min and labeled with [^{35}S]-

methionine at the same temperature for an additional 30 min. The results showed that three HMW-hsps, hsp75, hsp70B, and hsp68, were synthesized at a significantly stimulated rate in stationary cultures (Fig. 7). The densitometric analysis showed that the relative synthesizing rate of two other HMW-hsps, hsp83, and hsp70A, increased in arrested cells by 31 and 86%, respectively (Table III). We have noted that GM1 cells

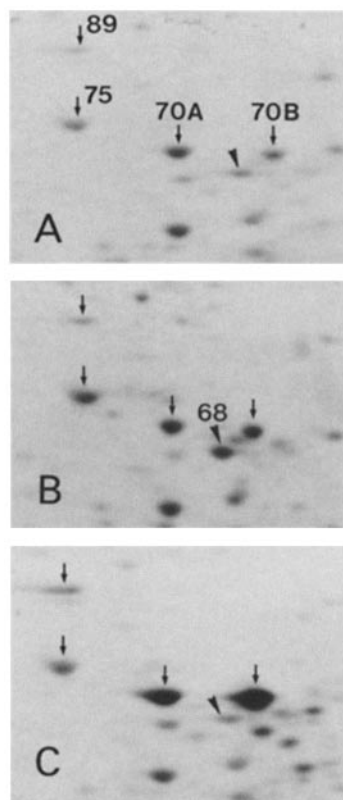


FIGURE 5 2D-NEPHGE/SDS-PAGE analysis of [^{35}S]-methionine-labeled proteins synthesized in growing and arrested CEF. Exponentially growing (A) and confluent (B) CEF cultures were labeled with [^{35}S]-methionine for 1 h at 37°C . (C) Exponentially growing cells were exposed to 45°C and labeled with [^{35}S]-methionine for 1 h. Arrows and an arrowhead indicate four hsps that are simultaneously G_0 proteins and a G_0 protein, respectively.

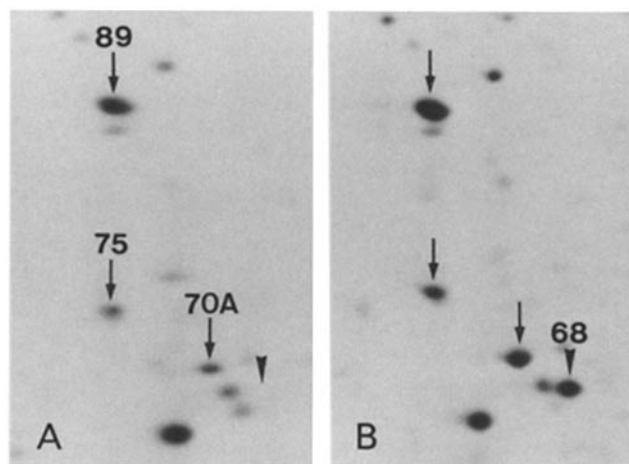


FIGURE 6 2D-NEPHGE/SDS PAGE analysis of [^{35}S]-methionine-labeled proteins synthesized in mouse T cells. Resting and proliferating mouse T lymphocytes were labeled with [^{35}S]-methionine for 1 h at 37°C . Whole proteins (1×10^6 dpm for each) were analyzed in two-dimensional gels. Time of SDS PAGE was twice as long so as to separate hsp89 from the spot that moved slightly faster than it. (A) Cells of day 2 culture stimulated with Con A (18). (B) Resting T lymphocytes that were freshly prepared from mouse spleens and purified as described in the text. Arrows and an arrowhead indicate three hsps that are simultaneously G_0 proteins and a G_0 protein, respectively. Hsp70B is absent in mouse lymphocytes.

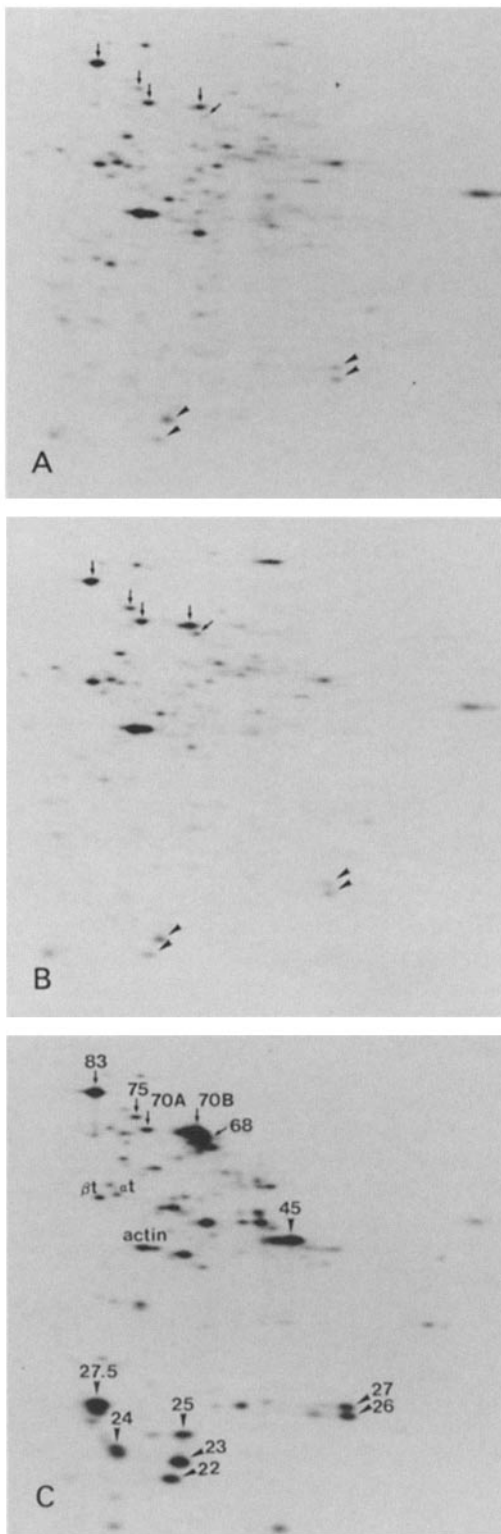


FIGURE 7 2D-NEPHGE/SDS PAGE analysis of [³⁵S]methionine-labeled proteins synthesized in *Drosophila* GM1 cells. (A) Exponentially growing cells at 25°C; (B) cells in stationary phase at 25°C; (C) Exponentially growing cells were heat-shocked at 36°C and labeled with [³⁵S]methionine as described in the text. Radiolabeled proteins (4×10^4 dpm) were analyzed for each. Arrows indicate hsp45 that are simultaneously G_0 proteins and arrowheads indicate hsp45 that are not G_0 proteins. Actin and tubulin (α t and β t) are also indicated.

synthesized at least four hsp45, hsp27.5, hsp25, and hsp24, in addition to common hsp45, including four LMW-hsps, hsp27, hsp26, hsp23, and hsp22, found in other cell

lines or salivary gland cells of the same species (4, 12, 28). The synthesis of seven LMW-hsps or hsp45 was not stimulated in stationary cultures.

Differing from yeast, the synthesis of most HMW-hsps is substantial even in exponentially growing cells of the three higher eucaryotic systems. Therefore, we are again confronted with the question as to persistence or induction of the synthesis of HMW-hsps in G_0 . We have first determined by densitometry the relative synthesizing rate of each HMW-hsp from fluorograms of two-dimensional gels on which radiolabeled proteins in a fixed radioactivity were loaded. The synthesizing rate of total proteins per milligram protein was also determined from the radioactivity of [³⁵S]methionine incorporated into trichloroacetic acid-precipitated materials. Comparisons were made between G_0 and growing cells for the synthesizing rate per milligram protein of each of the HMW-hsps.

The results shown in Table III indicate (a) the synthesizing rate of most major proteins, e. g., actin, decreased in G_0 cells in proportion to a reduction in the synthesizing rate of total proteins; (b) a proportion of radioactivity incorporated into any HMW-hsp to that incorporated into total proteins during the pulse was greater in G_0 cells than in growing cells; and (c) the synthesizing rate of hsp75, hsp70B, hsp68 (for *Drosophila* cells) and P68 (for chick and mouse cells) per milligram protein was greater in G_0 cells than in growing cells whereas that of hsp89/83 and hsp70A (except for mouse T lymphocytes) decreased in G_0 cells. We concluded, therefore, that at least hsp75 and hsp70B (also hsp68 in *Drosophila*) are specifically induced proteins in G_0 . We could not determine whether the synthesis of hsp89/83 and hsp70A persists in G_0 or alternatively is induced nevertheless the induction was offset by the decrease in the synthesis of total proteins. All these proteins are tentatively included in G_0 proteins of higher eucaryotes.

A list of all the G_0 and heat shock proteins found with cells of four eucaryotes is shown in Table IV.

DISCUSSION

G_0 Proteins and Heat Shock Proteins

Proteins that are specifically or preferentially synthesized during the transition from G_0 to S or G_1 or S have been studied by a number of investigators with respect to their possible functions in the initiation of growth (9, 34, 35, 38). Similarly, the investigation of proteins that are specifically synthesized during the reverse transition from G_1 to G_0 would be important because the function of these proteins might be essential for arrest in G_0 . So far, the latter investigation has not been performed, however.

Using a system with yeast cells, we have found that cells entered G_0 when the process of the cell cycle was appropriately blocked in G_1 , and detected at least nine G_0 -induced proteins in the yeast. Unexpectedly, six out of the nine G_0 proteins were found identical to those proteins detected as hsp45 of the same organism (Table I). This result suggests that the synthesis of the same polypeptides was induced by two different means, arrest in G_0 and heat shock. However, the duration of the synthesis was longer in G_0 than by heat shock (Table I). Therefore, the induction of six G_0 (and heat shock) proteins and the induction of the other simple hsp45 were distinctively detected even when cells of a temperature-sensitive mutant, for instance, *cdc25*, were incubated at nonpermissive temperature.

It has been widely accepted that both CEF in confluent culture and lymphocytes freshly prepared from spleens of

TABLE III
Synthesis of Hsps and Actin in Growing and G₀ Cells

| Cells | Growth phase | A: Rate of total protein synthesis/mg protein* | B: Rate of individual protein synthesis/rate of total protein synthesis [†] | | | | | |
|-----------------------------|--------------|--|--|---------------|----------------|---------------|--------------------------|---------------|
| | | | hsp89/83 | hsp75 | hsp70A | hsp70B | p68 [‡] (hsp68) | Actin |
| Chick embryonic fibroblasts | Exponential | 1.00 | 0.031 | 0.094 | 0.27 | 0.10 | 0.039 | 15 |
| | Confluent | 0.38 | 0.046 (0.56) [§] | 0.40 (1.6) | 0.65 (0.91) | 0.61 (2.3) | 0.74 (7.2) | 19 (0.48) |
| Mouse T lymphocytes | Growing | 1.00 | 0.24 | 0.028 | 0.052 | — | 0.001 | 12 |
| | Resting | 0.20 | 0.76 (0.63) | 0.21 (1.5) | 0.43 (1.7) | — | 3.5 (700) | 14 (0.23) |
| <i>Drosophila</i> GM1 | Exponential | 1.00 | 3.6 | 0.17 | 0.97 | 0.80 | 0.070 | 8.1 |
| | Stationary | 0.43 | 4.7 (0.56) | 0.87 (2.2) | 1.8 (0.80) | 3.7 (2.0) | 0.49 (3.0) | 8.7 (0.46) |

* Radioactivity of [³⁵S]methionine incorporated into trichloroacetic acid-insoluble materials/mg total proteins. The value for growing cells is expressed as 1.00 and the value for G₀ cells is expressed as a relative value to that of growing cells.

† The synthesizing rate of individual proteins was estimated from the results of two-dimensional gel electrophoresis shown in Figs. 5-7 as described in the legend to Table II.

‡ A G₀-protein, p68 was identified as a hsp in *Drosophila* cells but not in chick and mouse cells.

§ The values in parentheses represent the ratio of the synthesizing rate of each protein per milligram total proteins in G₀ cells to that in growing cells, and were calculated by A of G₀ cells × $\frac{B \text{ of } G_0 \text{ cells}}{B \text{ of growing cells}}$.

TABLE IV
List of Hsps Synthesized in G₀ Cells

| Cells | Hsps |
|--------------------------------|--|
| Yeast (<i>S. cerevisiae</i>) | hsp89, hsp86, hsp75, hsp71, hsp48A, hsp46A |
| Chick embryonic fibroblasts | hsp89, hsp75, hsp70A, hsp70B |
| Mouse T lymphocytes | hsp89, hsp75, hsp70A |
| GM1 (<i>D. melanogaster</i>) | hsp83, hsp75, hsp70A, hsp70B, hsp68 |

unimmunized mice are resting in G₀ of the cell cycle (3, 18). Although the background level of the synthesis of HMW-hsps is not negligible in growing CEF and lymphocytes, these proteins are synthesized at a relatively stimulated rate in G₀ or in heat-shocked cells (Figs. 5 and 6, and Table III). A similar observation was made with *Drosophila* cells. Therefore, the results seem to suggest a rule commonly held in eucaryotes that many, if not all, of HMW-hsps were preferentially synthesized in G₀.

Induction Mechanism and Function of Hsps in G₀

The synthesis of hsp is induced by various treatments or agents besides heat shock, including uncouplers of oxidative phosphorylation, inhibitors of electron transports, recovery from anoxia (1), transition metals, sulfhydryl reagents (20), amino acid analogues (16), trauma (8), and ethanol (21, 22). Although the heat shock response could have evolved differently in homeotherms and heterotherms, organisms may be commonly endowed with a family of hsp as a defensive mechanism against these unusual and severe conditions so that cells can survive in these environments (1). In fact, evidence has been provided in a bacterium, *Escherichia coli*, that the synthesis of hsp is essential for growth at elevated temperature (39). Furthermore, it was shown with *Drosophila* (29), yeast (26), *Dictyostelium* (24), and Chinese hamster fibroblasts (21, 22) that an accumulation of hsp is positively correlated with the degree to which cells are resistant to high temperature. In the context, the observation that hsp were preferentially synthesized in G₀ seems to be compatible with

the finding made by Schenberg-Frascino and Moustacchi (36) that stationary yeast cells were much more resistant against thermal killing than exponentially growing cells. All of the above inducing agents and treatments stopped cells from growing, but none caused G₀ arrest.

In contrast to the above unusual and undesirable conditions for cells where hsp were induced, those that led cells to enter G₀ do not seem widely different from physiological conditions for cells. For instance, deprivation of growth factors or mitogen from medium appears to mimic the in vivo regenerating system of G₀ cells in higher organisms (18).

The synthesis of hsp induced in G₀ differs from that induced by heat shock in two respects. First, the duration of hsp synthesis at the stimulated rate continued longer in G₀ cells than in heat-shocked cells (Figs. 1 and 2, and Table I). Second, an increase in the synthesis of LMW-hsps have never been observed in cells that were arrested in G₀ (Table I, and Figs. 1 and 7). Previous studies have shown that different hsp had different induction characteristics with respect to the optimal temperature, the range of effective temperature, and the kinetics of their induction (23). In addition, a common nucleotide sequence was detected in the HMW-hsp genes around 70 nucleotides upstream from the TATA box but not in the hsp26 gene (10). This suggests that mRNA synthesis of HMW-hsps is regulated by a mechanism different from the mechanism that operates in the induction of LMW-hsps. In agreement with this, it was shown that treatment of cultured *Drosophila* cells with ecdysterone induced the synthesis of LMW-hsps but not that of HMW-hsps (12). We have observed that the synthesis of LMW-hsps was induced and that the degree to which HMW-hsps had been expressed was altered when G₀ CEF cells, resting mouse lymphocytes, or stationary *Drosophila* cells were heat-shocked (unpublished observations). Therefore, the mechanism of the hsp induction operating in G₀ and that operating in the heat shock would be different, although they might share common parts.

The results presented in this report clearly show that transition of growing eucaryotic cells to G₀ was accompanied with the synthesis of HMW-hsps. Although data are not available that indicate a causal relationship between the synthesis of hsp and G₀ arrest, the results seem to imply two possibilities.

First, HMW-hsps may function in cellular events required for the transition to G_0 and/or maintaining G_0 . However, the synthesis of hsps could not be the sole cause of the G_0 transition because the induction of hsp synthesis simply by heat shock did not bring cells to enter G_0 . Conditions under which cells entered G_0 induced the durable synthesis of HMW-hsps, whereas heat shock induced the transient synthesis of both HMW- and LMW-hsps. In this respect, it would be of interest that a transient G_1 arrest was induced when a nontemperature-sensitive strain of yeast was exposed to high temperature (14). Therefore, the continuous expression of certain, if not all, hsps may be necessary for sustaining the G_0 state.

Second, it is also possible that the durable synthesis of HMW-hsps might be one of the cellular events that results from G_0 . The synthesis of most proteins, including repressor-like molecules of the hsp genes, is reduced in G_0 , which then induces the expression of these genes. Cytosols of heat-shocked *Drosophila* cells appeared to contain substances which induce heat shock puffs in isolated polytene nuclei (6, 7). The second possibility would be unlikely, therefore, if the expression of hsp genes is positively controlled.

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