Control of hsp70 RNA Levels in Human Lymphocytes

Leszek Kaczmarek,* Bruno Calabretta,* Hung-Teh Kao,‡ Nathaniel Heintz,§
Joseph Nevins,‡ and Renato Baserga*

*Department of Pathology, Temple University Medical School, Philadelphia, Pennsylvania 19140; and
§Laboratory of Biochemistry and Molecular Biology, and ‡Howard Hughes Medical Institute, The Rockefeller University,
New York, New York 10021

Abstract. The expression of a *hsp70* gene in human cells has previously been shown to be related to the growth state of the cells. As an alternative to in vitro synchronization procedures, we have measured steady-state levels of the RNA for a heat-shock protein 70 (hsp70) in human peripheral blood mononuclear cells (PBMC) that are naturally quiescent in a G_0 state. The probe used recognized, on RNA blots, one single band. The levels of this hsp70 RNA are elevated in circulating PBMC and decrease when the cells are incubated with serum, or phytohemagglutinin, or simply when they are incubated in culture medium. The levels of hsp70 RNA decrease within 30 min after in vitro culture, and are accompanied by an increase in the levels of c-*fos* RNA. These findings, together with other recent reports in the literature, suggest a possible role of the hsp70 proteins in the regulation of cell growth.

THE heat shock proteins (hsps)[1] are a group of highly evolutionary conserved proteins (Ritossa, 1964; review in Schlesinger et al., 1982) whose amounts are dramatically increased when cells are treated with a variety of stresses, including elevation of temperature (Ashburner and Bonner, 1978), anoxia, trauma (Currie and White, 1981), and administration of certain chemicals (Li, 1983). The increased expression of hsps has been attributed to the accumulation of abnormal cellular proteins (Kelley and Schlesinger, 1978).

One of the best characterized hsps is hsp70 (a protein of 70,000 mol wt). Recently, several reports have been published relating the expression of hsp70 to cell proliferation. While we recognize that the hsp70 consists of a gene family that is closely related in sequence, it is still true that the products of this gene family are often related to growth conditions. Thus, analysis of hsp70 expression in synchronized cultures of HeLa cells showed that the amount of a hsp70 mRNA reached its maximum during the G_2 phase of the cell cycle although maximal transcription was earlier, probably in late S phase (Kao et al., 1985). Similarly, Wu and Morimoto (1985) observed an induction of *hsp70* gene expression when serum-starved human HeLa and 293 cells were stimulated to proliferate by serum. Moreover, the expression of hsp70 is induced by adenovirus infection (Nevins, 1982; Kao and Nevins, 1983; Imperiale et al., 1984), and adenovirus infection of quiescent nonpermissive fibroblasts results in the expression of several late G_1/S phase genes (Liu et al., 1985). Iida and Yahara (1984a) analyzed proteins

specific for quiescence in yeasts and found that hsps with high molecular weight (HMW) comprise a significant portion of such proteins. When those authors extended their studies to cells of higher eukaryotes, they found that most of HMW hsps are present in elevated amounts in growth-arrested (G_0) cells when compared with proliferating cells (Iida and Yahara, 1984b). Among the proteins tested, hsp70 was found to be present in increased amounts in quiescent chicken fibroblasts as well as in mouse T lymphocytes (Iida and Yahara, 1984b).

To investigate these apparently contradictory results relating HMW hsps (including hsp70) on the one side to the G_0 phase and, on the other side, to the S/G_2 phases of the cell cycle, we investigated the mRNA levels of hsp70 in human peripheral blood mononuclear cells (PBMC). These cells seem to be particularly suitable for such a purpose. In the first place they are physiologically quiescent (G_0) when taken directly from peripheral blood. Secondly, they can be easily stimulated to proliferate with T cell-specific mitogens like phytohemagglutinin (PHA). Thirdly, it is possible to obtain and analyze pure populations of T lymphocytes responding to complete or incomplete mitogenic stimuli resulting either in cellular proliferation or in cellular growth in size without replication of nuclear DNA (Maizel et al., 1981; Mercer and Baserga, 1985). Thus, these cells allow an analysis of *hsp70* gene expression in relation to cell growth, but from a different perspective than synchronized HeLa cells. Our results indeed indicate that hsp70 mRNA levels are elevated in PBMC but rapidly decline when these cells are placed in culture. Since the probe we used recognized, in RNA blots, a single band, and since this band decreased in intensity or disappeared after stimulation, one can state un-

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1. *Abbreviations used in this paper*: HMW, high molecular weight; hsp(s), heat shock protein(s); IL-2, interleukin 2; PBMC, human peripheral blood mononuclear cells; PHA, phytohemagglutinin.

equivocally that the hsp70 RNA recognized by our probe is growth-regulated. On the other hand, we cannot say at this point which, among the *hsp70* genes, is growth-regulated.

Materials and Methods

Isolation and Culture Conditions of PBMC

The procedures to isolate PBMC, purify T cells, and culture PBMC and T lymphocytes in the absence or presence of interleukin 2 (IL-2 [kind gift of Cetus Corp., Emeryville, CA]) have been described previously (Kaczmarek et al., 1985a, b). Similarly, the procedures for monitoring the levels of mitogenic stimulation in cultures by autoradiography of [³H]thymidine-labeled cells were described in a previous paper (Mercer and Baserga, 1985).

Analysis of Gene Expression

The extraction of total cellular RNA from lymphocytes has been described elsewhere (Calabretta et al., 1985).

Northern Blot Analysis and Hybridization Conditions (Thomas, 1980)

The following gene probes were used (a) pURHS, which is a cDNA clone of the human hsp70 mRNA (Kao and Nevins, 1983); (b) pFo422 (a kind gift of Dr. G. Stein, University of Florida), which has an insert that encodes genomic sequences of human histone H3 and (c) pHe7, which contains a cDNA insert recognizing a non-cell-cycle-dependent mRNA (Babich et al., 1983). 20 μg of total RNA was used for each lane.

Results

In previous reports (Kaczmarek et al., 1985a, b), we described in detail the conditions used in one of our laboratories for the stimulation of PBMC by PHA. When PBMC, freshly isolated on Ficoll gradients, are incubated in RPMI 1640 medium plus 5% FCS and 10 μg/ml PHA, from 50 to 70% of the lymphocytes enter S phase, predominantly between 48 and 66 h of culture. The stimulation of cellular DNA synthesis by PHA is accompanied by an increase in steady-state levels of cytoplasmic mRNAs of a variety of growth-related genes including c-*myc* (Kaczmarek et al., 1985a), as also reported by other investigators using slightly different conditions (Kelly et al., 1983; Reed et al., 1986). The RNA for the histone H3 gene, whose expression is strictly S phase-related (Plumb et al., 1983), is first detectable 36 h after stimulation of PBMC and reaches its maximum at ~66 h post-stimulation (Kaczmarek et al., 1985a). If PHA is omitted, <0.1% of the cells enter S phase in up to 84 h of incubation (Kaczmarek et al., 1985a), and there is no increase in the expression of growth-related genes (Kaczmarek et al., 1985a). If macrophages are depleted from PBMC, the addition of PHA causes an increase in size of T lymphocytes but no DNA synthesis (Maizel et al., 1981; Mercer and Baserga, 1985). Under these conditions, the mRNA levels of growth-related genes also fail to increase (Kaczmarek et al., 1985a, b; Mercer and Baserga, 1985), with two exceptions, i.e. the IL-2 receptor mRNA and the RNA cognate to the cell cycle cDNA clone called 2F1 (Hirschhorn et al., 1984).

The percentage of PBMC labeled by [³H]thymidine under various condition was constantly monitored by autoradiography throughout the present experiments, but the data are not shown because they were not different from those repeatedly published by ours and other laboratories.
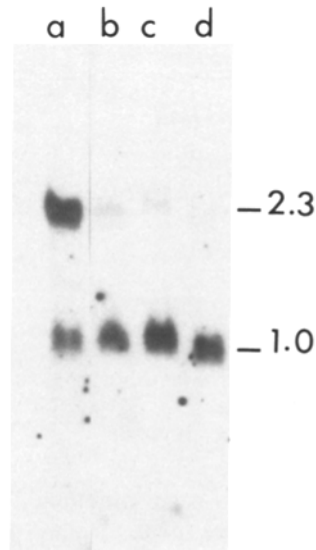

Figure 1. Levels of hps70 RNA in PBMC stimulated with PHA. RNA was prepared as described in Methods and Materials from freshly isolated or PHA-stimulated PBMC. RNA blots were carried out as described (Thomas, 1980). The probes used were: hsp70 (upper band) and *pHe7*, which hybridizes to an RNA whose expression is not cell cycle–dependent (lower band). Lanes: a, freshly isolated PBMC; b, c, and d, 22, 36, and 66 h, respectively, after PHA stimulation.

hsp70 RNA Levels in PHA-stimulated PBMC

Fig. 1 shows an RNA blot of PBMC, either freshly isolated (lane a) or at various times after PHA-stimulation (lanes b–d). The blot was hybridized to nick-translated hsp70 cDNA and the control, non-cell-cycle-regulated gene, *pHe7* (Babich et al., 1983). The hsp70 RNA is clearly detectable in lane a as a band 2.7 kb in size, which decreases in intensity and eventually disappears in PHA-stimulated PBMC. The band corresponding to pHe7 RNA, 1.0 kb in size, has a constant intensity, indicating that the amount of RNA analyzed and transferred to the filter is roughly the same in each lane. It also indicates that the decrease in hsp70 RNA is not merely a reflection of a generalized decline in mRNA levels.

Fig. 2 shows the level of hsp70 mRNA under different conditions of cell culture. The hsp70 RNA is clearly detectable in unstimulated PBMC (lane a), but not in PBMC stimulated with PHA for 3, 12, or 22 h (lanes b, d, and g). The hsp70 RNA is not detectable in MDC cultures, i.e., macrophages depleted cultures stimulated by PHA independently of the presence (lanes f and i) or absence (lanes c, e, and h) of IL-2. Since, in the absence of IL-2, macrophage-depleted lymphocytes do not enter S phase (see above), the results in Fig. 2 are the first indication that the disappearance of hsp70 RNA in PHA-treated PBMC is not strictly correlated to the stimulation of cellular DNA synthesis.

Elevated hsp70 RNA Levels Are Not Due to Stress

Since the hsp70 promoter is responsive to stress as well as to heat shock (Kelley and Schlesinger, 1978; Li, 1983), it seemed possible that the presence of high levels of hsp70 RNA in PBMC was due to the potential stress of the purification procedure through Ficoll. Accordingly, after stimulation with PHA for 6 h, we repurified the original PBMC through Ficoll. RNA was prepared from cells before and after repurification and the levels of hsp70 RNA were assayed as usual. The results are shown in Fig. 3. Again, PHA-stimulation of PBMC results in a decline of the hsp70 RNA (lanes a and b). If the PBMC, 6 h after PHA, were again passed through a Ficoll column, there was no reappearance of the hsp70 RNA (lane c). However, the PBMC are still

Figure 2. Levels of hsp RNA in PBMC under various culture conditions. Lane a, freshly isolated PBMC; lanes b, d, and g, PBMC stimulated with PHA for 3, 12, and 22 h; lanes c, e, and h, macrophage-depleted cultures, unstimulated and 12 and 22 h after PHA stimulation, respectively (no IL-2); lanes f and i, macrophage-depleted PBMC, PHA-stimulated for 12 and 22 h, respectively, in the presence of IL-2. Other conditions as in Fig. 1.

responsive to a heat shock as shown in Fig. 4. Again, hsp70 RNA was virtually nondetectable when PBMC were stimulated with PHA. If the PHA-stimulated PBMC are subjected to a heat shock (lane c), hsp70 RNA increases to significant levels. It seems, therefore, that the high level of hsp70 RNA in G_0 lymphocytes is not due to the stress of purification through the Ficoll column.

We then examined the components, in the incubation mixture, that cause the disappearance of the hsp70 RNA from G_0 lymphocytes. The results are shown in Fig. 5. Neither PHA nor FCS (lanes b and c) are necessary. As clearly shown in lane c, incubation of PBMC in RPM1 1640 medium (serum-free, without PHA) is sufficient to cause the disappearance of hsp70 RNA (lane c).

The Disappearance of hsp70 RNA Is Accompanied by the Induction of c-fos RNA

It is possible that the incubation of PBMC in serum-free media may, by itself, modify the physiological state of the cells, without inducing cellular DNA synthesis. Several instances are known in which incomplete mitogens can change the physiological state of G_0 cells without inducing cellular DNA synthesis (Todaro et al., 1965; Burk, 1970; Bombik and Baserga, 1974; Kaczmarek et al., 1985c). One of the earliest markers of a change in resting cells exposed to growth factors is the expression of the proto-oncogene c-fos. c-fos RNA is
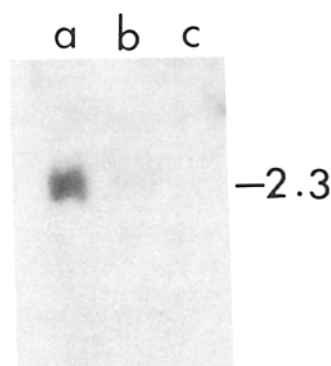

Figure 3. Levels of hsp70 RNA in PBMC passed through a Ficoll column. Lane a, PBMC freshly isolated through a Ficoll column; b, same 6 h after PHA stimulation; c, same 6 h after PHA stimulation and passed again through a Ficoll column. Other conditions as in Fig. 1.
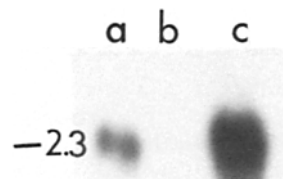

Figure 4. Levels of hsp70 RNA in heat-shocked PBMC. Lane a, freshly isolated PBMC; b, PBMC 6 h after PHA stimulation; c, same as b, except the cells were subjected to a heat shock (30 min at 42°C). Other conditions as in Fig. 1.

usually undetectable in G_0 cells, but it is quickly induced by growth factors, as early as 5 min after exposure (Greenberg and Ziff, 1984; Kruijer et al., 1984; Muller et al., 1984). It precedes the appearance of c-myc RNA and of other RNAs transcribed from growth-regulated genes. This is also true of PHA-stimulated PBMC (Reed et al., 1986). We therefore investigated the expression of c-fos in PBMC incubated in serum-free, PHA-free medium. For this purpose, we used whole blood cells (from a buffy coat) without previous purification through a Ficoll gradient. The cells were then incubated in RPM1 1640 medium only and RNA was prepared 30 min after incubation. The results of a Northern analysis of this RNA are shown in Fig. 6. Within 30 min of incubation, the amount of hsp70 RNA has decreased sharply (lanes a and b). Coincident with this decline in hsp70 RNA is an increase in c-fos RNA (lanes c and d).

Therefore, whatever the specific conditions are that result in a decline of hsp70 RNA when PBMC are incubated in culture media, these conditions also result in an induction of c-fos RNA, which is considered a marker for the earli-
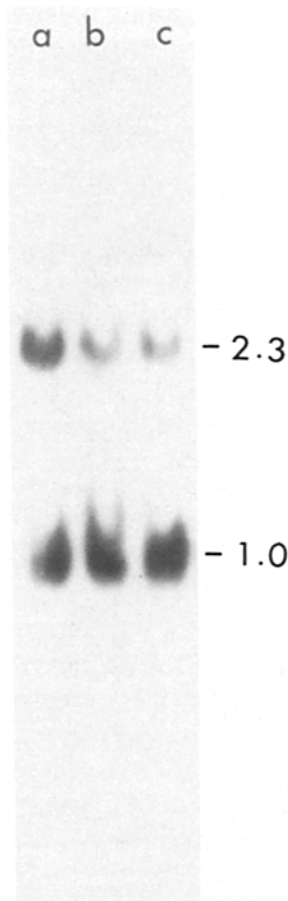

Figure 5. Effect of medium components on the levels of hsp70 RNA in human PBMC. Lane a, freshly isolated PBMC; b, PBMC incubated in RPMI 1640 medium plus 10% fetal calf serum; c, no additions. Other conditions as in Fig. 1.
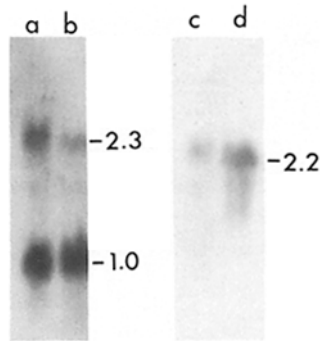
Figure 6. Effect of incubation in medium on the levels of hsp70 and c-*fos* RNAs in PBMC. Blood cells from a buffy coat were either untreated (lane *a*) or incubated in serum-free, PHA-free medium for 30 min (lane *b*). RNA was isolated, and the RNA blots were hybridized simultaneously to the probes for hsp70 (upper band) and *pHe7* (lower band). Lanes *c* and *d* are of the same blot, except that the probe used was c-*fos*. Experimental details are given in Methods and Materials.

est events that occur when G_0 cells are exposed to growth factors.

Discussion

Previous experiments have indicated that the expression of one of the human *hsp70* genes is regulated during the cell cycle. Assays using either drug-synchronized HeLa cells (Kao et al., 1985) as well as HeLa or 293 cells synchronized by serum deprivation (Wu and Morimoto, 1985) have shown that the levels of hsp70 RNA increase markedly upon release of the cells into the growing state and that the increase was due to an activation of transcription of the gene. Furthermore, the activation does not appear to be indirectly due to a stress response related to the procedure of synchronization, since an *hsp70* gene deleted of the stress response element retains the response to serum (Wu et al., 1986). Therefore, independent of the regulation of these genes by heat shock, there is also regulation by growth factors.

In contrast to these previous results we now find high levels of hsp70 RNA in G_0 lymphocytes, and when these cells take the initial step toward proliferation, the hsp70 RNA declines to low levels. How can this result be explained in light of the previous findings? Obviously, one possibility is that the regulation of the *hsp70* genes is different in HeLa cells and lymphocytes. It is quite possible that the expression of the *hsp70* gene, either in terms of RNA or protein levels, varies from one cell type to another. In some cells, it may be associated with cell proliferation, in other with quiescence or even differentiation. This in fact is the case with some proto-oncogenes, which in some cells are expressed when mitogenic stimuli are applied (Greenberg and Ziff, 1984; Kruijer et al., 1984), while in other types of cells they are induced after differentiating stimuli (Kruijer et al., 1985; Greenberg et al., 1985) or reach high levels in postmitotic cells like neurones (Brugge et al., 1985) and blood platelets (Golden et al., 1986). There are also alternative explanations.

One possibility is that there might be a link between the accumulation seen previously in G_2 phase cells and the high levels seen here in G_0 PBMC. There would appear to be two possible explanations for the presence of gene products specifically in G_0 cells. First, the genes could be activated when cells enter G_0 and thus the gene products would only be found in cells in this phase. Alternatively, when cells cease to proliferate and leave the cell cycle, there could be a retention of certain gene products that accumulated at the end of the previous cell cycle. These gene products would thus be characteristic of G_0 cells as well as of G_2 or M phase cycling cells.

The second possibility is consistent with the present results and previous data for the expression of the *hsp70* gene. We would propose that the high level of hsp70 RNA found in G_0 lymphocytes might be a carry-over from the previous G_2 accumulation of hsp70 RNA. When the G_0 lymphocytes are then stimulated to proliferate and reenter the cell cycle, the hsp70 RNA declines as it might normally after G_2 in cycling cells.

A third explanation is based on the recognition that the *hsp70* gene actually consists of a gene family. It is conceivable that the *hsp70* gene active in G_0 lymphocytes may be a different one from the *hsp70* gene, whose expression reaches its peak in the G_2 phase of HeLa cells. This is a reasonable possibility. However, in the present experiments, stimulation of PBMC results in the decrease or disappearance of the only RNA band that is recognized by our probe. Whatever *hsp70* gene transcript is recognized by our probe, we can say that it is highly expressed in G_0 lymphocytes and is not expressed (or markedly decreased) in proliferating cells, which in itself is an intriguing observation.

An important observation emerging from the present experiments is that incubation of PBMC in culture media results not only in the decline of hsp70 RNA levels but also in an increase in c-*fos* RNA. Although c-*fos* is the cellular equivalent of a retroviral transforming gene and is known to be induced in G_0 cells by growth factors (Greenberg and Ziff, 1984; Kruijer et al., 1984; Muller et al., 1984), it is also induced in the absence of cellular proliferation. Thus, it is induced by epidermal growth factor in A431 cells (Bravo et al., 1985), in which epidermal growth factor does not induce cellular DNA synthesis, and it also markedly increased when PC12 cells are induced to differentiate by nerve growth factor (Greenberg et al., 1985; Kruijer et al., 1985). In our experiments, c-*fos* is induced by incubation in mitogen-free media. The most logical interpretation is that incubation of PBMC in serum-free media causes the cells to leave G_0 and enter G_1 without progressing to S phase. There are several instances in the literature in which exposure of G_0 cells to certain growth factors initiates some early steps in cell cycle progression, without cellular DNA synthesis (Burk, 1970; Bombik and Baserga, 1974; Kaczmarek et al., 1985c, 1986). The most dramatic example, of course, is given by the experiments with fibroblasts in culture that led to the formulation of the competence–progression theory (Stiles et al., 1979; for a review see Scher et al., 1979), but a similar situation can also be found in stimulated T and B lymphocytes (Maizel et al., 1981; Melchers and Lernhardt, 1985). Indeed, the experiments in the present paper, in which purified T lymphocytes respond to PHA by growing in size but without entering DNA synthesis (Maizel et al., 1981; Mercer and Baserga, 1985) are a good example of exit from G_0 without S phase.

From several accounts the expression of the *hsp70* gene appears to be subject to control in relation to cell growth. Does this mean that the *hsp70* gene product plays a role in cell growth? There is in fact evidence for such a role from genetic experiments in yeast. Multiple *hsp70* genes are found in yeast

as is the case in most other organisms. Deletion of one of several of the genes produced no apparent phenotype; however, deletion of two genes resulted in impaired growth at physiological temperature, indicating a requirement for these gene products in normal cell growth (Craig and Jacobsen, 1984). Whether there is also an important role for the protein(s) in mammalian cells is unclear. However, given the striking control of the gene in relation to cell growth, under two quite different experimental conditions, it seems most likely that the protein is involved in the growth of cells. An attractive possibility is that the removal of a *hsp70* gene product is a necesary requirement for cells to reenter the cell cycle.

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