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Expression of human HSP70 during the synthetic phase of the cell cycle

(growth regulation/heat shock/human cells)

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ABSTRACT **Expression of the major heat shock and stress-induced protein, HSP70, is under complex regulatory control in human cells. In addition to being induced by physiological stress such as heat shock or transition metals, the *HSP70* gene is induced by serum stimulation and immortalizing products of the adenovirus E1A 13S and polyoma large tumor antigen genes. Here we show that expression of the human *HSP70* gene is tightly regulated during the cell cycle. Using selective mitotic detachment, a noninductive method to obtain synchronous populations of HeLa cells, we show that levels of HSP70 mRNA rapidly increase 10- to 15-fold upon entry into S phase and decline by late S and G_2. A transient increase in HSP70 synthesis is detected during early S phase. The subcellular localization of HSP70 varies throughout the cell cycle; the protein is diffusely distributed in the nucleus and cytoplasm in G_1, localized in the nucleus in S, and again diffusely distributed in G_2 cells. We suggest that the temporal pattern of HSP70 expression during S phase, the nuclear localization, and activation by trans-acting immortalizing proteins indicate a role for HSP70 in the nucleus of replicating cells.**

The cellular transition from the resting state to the growing state requires expression of certain genes whose products regulate critical events during the G_0 and G_1 phases of the cell cycle. Serum stimulation has been widely used as a method to identify and examine genes that are growth-regulated. The addition of fresh serum to quiescent mammalian cells activates the expression of a family of growth-regulated genes, including c-*fos*, c-*myc*, β-interferon, proliferin, p53, and *HSP70* (1–10). Many of these genes are activated by specific growth factors such as platelet-derived growth factor or epidermal growth factor, suggesting the possibility that their expression may be regulated by common mechanisms (1, 2, 7).

Workers in our laboratory have studied the expression of the major heat shock protein, HSP70, in human cells. An intriguing relationship between the expression of heat shock proteins and cellular transformation has been established. HSP70 is expressed at high levels in transformed mammalian cells and is induced in cells infected with DNA tumor viruses (11–15). Expression from the HSP70 promoter is induced by the E1A 13S product of adenovirus and by large tumor antigen of polyoma (15, 16). These transforming proteins are capable of immortalizing cells in culture (17–20) and stimulating transcription in trans (reviewed in ref. 21).

We have demonstrated (10) that *HSP70* gene expression in a transformed human cell line, HeLa, was induced by serum stimulation. To examine whether this corresponds to expression at a specific point during the cell cycle or to activation by specific growth factors, we have used a noninductive method—that of selective mitotic detachment—to obtain synchronous populations of cells. We show that maximal levels of HSP70 mRNA are detected in S phase at the peak of DNA synthesis. HSP70 protein synthesis increases transiently at this point and increased levels of HSP70 are detected in the nucleus of S-phase cells.

METHODS

Cell Culture and Synchronization. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Conditioned medium was harvested from cells following 48 hr of growth from 20% to 80% confluence and was clarified by centrifugation. Synchronized populations of cells were obtained by selectively harvesting mitotic cells and replating in conditioned medium (22). To determine the position in the cell cycle at the indicated times after plating, 2×10^4 cells were incubated with [^{3}H]thymidine (2 μCi/ml; 1 Ci = 37 GBq) for 30 min, and incorporation into DNA was determined by precipitation with 10% trichloroacetic acid. The extent of cell synchrony was also measured from autoradiography of cells incubated with [^{3}H]thymidine (3 μCi/ml) for 60 min.

Analysis of HSP70 mRNA Levels. Total cytoplasmic RNA was prepared from synchronized populations of cells. The level of HSP70 mRNA was analyzed by hybridization of cytoplasmic RNA to a 300-nucleotide ^{32}P-labeled template of the human *HSP70* gene (15), which protects the 5′-terminal 150 nucleotides of HSP70 mRNA, followed by S1 nuclease digestion and analysis by electrophoresis in polyacrylamide/urea gels (23).

Synthesis and Levels of HSP70 Protein. Synchronized cells growing on 60-mm plastic dishes were washed and labeled for 1 hr with [^{35}S]methionine (100 μCi/ml) in DMEM minus methionine. Asynchronous cells grown at 37°C or exposed to 42°C for 90 min were similarly labeled, washed in phosphate-buffered saline (140 mM NaCl/2.7 mM KCl/4.27 mM Na_2HPO_4/1.5 mM KH_2PO_4), and lysed in urea sample buffer. Isoelectric focusing in the first dimension (ampholytes, pH 5–7) and NaDodSO$_4$/PAGE in the second dimension (12.5% acrylamide) were performed (24).

The levels of HSP70 protein in synchronous populations were determined by immunoblot analysis (25). Equivalent numbers of cells at different points in the cell cycle were lysed in Laemmli sample buffer (26), separated by NaDodSO$_4$/PAGE, and blotted to nitrocellulose. The filter was incubated with a monoclonal C92 antibody (1:100) shown to be specific only for HSP70 (27) and the reaction was followed by iodinated goat anti-mouse antibody.

Indirect Immunofluorescence. The intracellular localization of HSP70 was determined by indirect immunofluorescence using monoclonal antibody C92. At the indicated times, cells were fixed and permeabilized by exposure to methanol at −20°C for 2 min and incubated with monoclonal antibody C92

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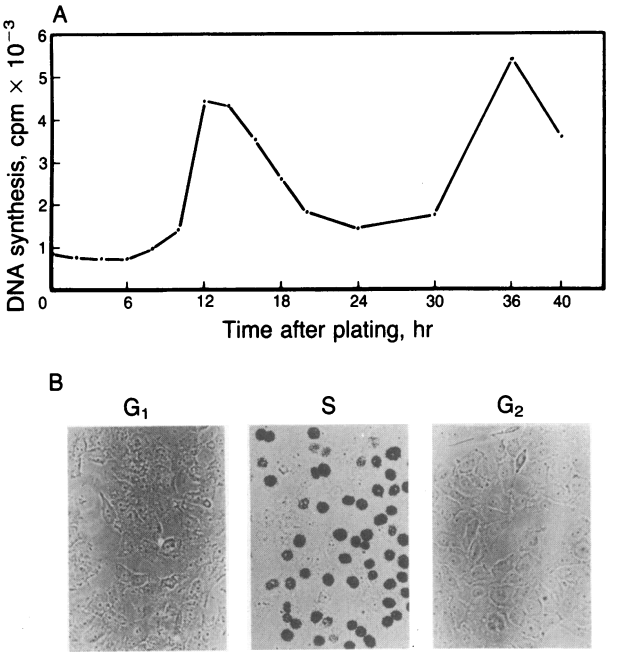
Abbreviation: HSP, heat shock protein.



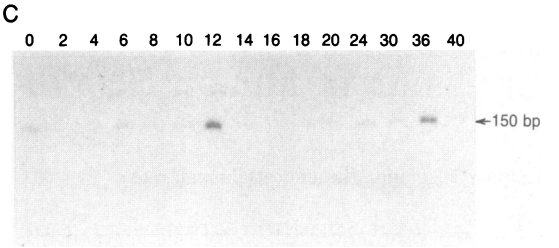


FIG. 1. Cell synchrony and levels of HSP70 mRNA. (*A*) DNA synthesis was measured by [^{3}H]thymidine incorporation at the indicated times after plating harvested mitotic HeLa cells. (*B*) Mitotic index of G_1, S, and G_2 populations of synchronized cells. Mitotic cells were plated in chamber slides and individual chambers were incubated with [^{3}H]thymidine during G_1, S, and G_2. Slides were dipped in Kodak NTB-2 emulsion diluted 1:1 with 0.6 M ammonium acetate (4-day exposure). (*C*) Relative levels of HSP70 mRNA as determined by S1 nuclease analysis. At the indicated times (hr) after plating harvested mitotic cells, cytoplasmic RNA was prepared. Equivalent amounts of RNA (10 μg) were hybridized to a 300-base-pair (bp) *HSP70* gene-specific template and digested with S1 nuclease. Protected fragments correspond to 150 bp at the 5′ terminus of the message and were analyzed by electrophoresis in 4% polyacrylamide/urea gels.

(1:50) followed by fluorescein-conjugated goat anti-mouse antibody (1:50). The slides were mounted and the cells were photographed with a Nikon fluorescent microscope.

RESULTS

HeLa Cell Synchrony and HSP70 mRNA Levels. We have shown (10) that transcription of the *HSP70* gene in human cells in culture is induced by serum stimulation. However, because growth of HeLa cells like other transformed cells is not dependent on high levels of exogenous serum, these results only suggest that expression of the *HSP70* gene is growth-regulated. To further examine the expression of *HSP70* during cell growth, we obtained synchronized populations of HeLa cells by a noninductive method—selective mitotic detachment. Mitotic cells were harvested and replated in conditioned medium to avoid serum stimulation. At various times after plating mitotic cells, a microtiter well was labeled with [^{3}H]thymidine to monitor DNA synthesis. The synthetic phase begins 11–12 hr after plating and continues for a period of 6–7 hr, after which the incorporation of [^{3}H]thymidine returns to background levels (Fig. 1*A*). Cells synchronized by selective mitotic detachment remain synchronous through a second round of DNA synthesis, which peaks 36 hr after the initial plating. We infer from the time between the two peaks of DNA synthesis that the length of the HeLa cell cycle is ≈24 hr. A second independent measure

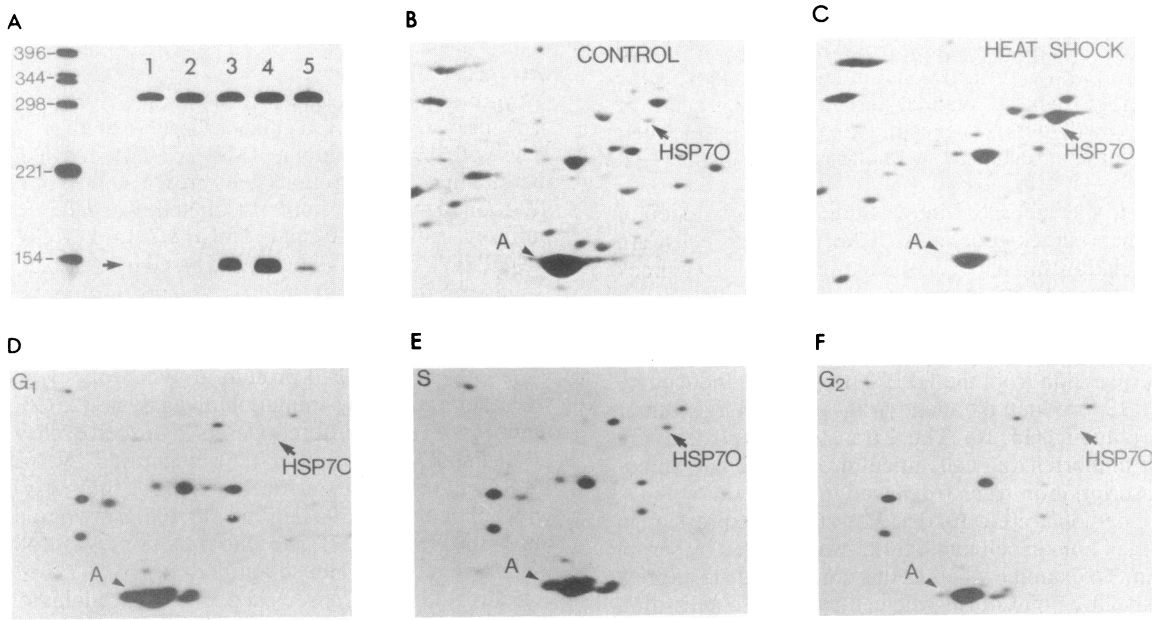


FIG. 2. Synthesis of HSP70 protein in synchronized HeLa cells. (*A*) HSP70 mRNA levels were determined by S1 nuclease protection. Cytoplasmic RNA was isolated from cells in G_1 (4 hr after plating mitotic cells; lane 2), S (11 and 12 hr after plating; lanes 3 and 4), and G_2 (22 hr after plating; lane 5) and hybridized to the template (lane 1) described above. Molecular size markers are indicated in the leftmost lane. (*B–F*) HSP70 protein synthesis was analyzed by two-dimensional gel electrophoresis. Asynchronous populations of cells grown at 37°C (*B*) or exposed to 42°C (*C*), and synchronized cells (37°C) in G_1 (*D*), S (*E*), and G_2 (*F*) were labeled with [^{35}S]methionine for 1 hr. Equivalent numbers of cells were loaded. HSP70 is indicated with an arrow; actin (A) is noted with an arrowhead.

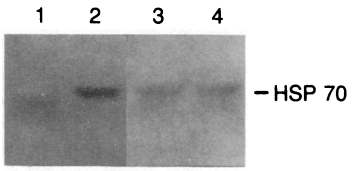


FIG. 3. Levels of HSP70 protein throughout the cell cycle by immunoblot analysis. Equal cell aliquots were lysed in sample buffer, electrophoretically separated by $NaDodSO_4$/PAGE, immunoblotted to nitrocellulose, and incubated with C92 antibody. The lanes correspond to synchronous populations of G_1 (lane 1), early S (lane 2), late S (lane 3), and G_2 (lane 4) cells.

of the level of cell synchrony is shown by autoradiography of a representative population of synchronized cells labeled with [^{3}H]thymidine in the G_1, S, and G_2 phases of the cell cycle. At the peak of DNA synthesis, 80–90% of the population shows grains over the nucleus, whereas in both G_1 and G_2 populations, incorporation of [^{3}H]thymidine is detected in <5% of the cells (Fig. 1*B*).

The levels of HSP70 mRNA in populations of synchronized cells were analyzed by the method of S1 nuclease protection using a HSP70 gene-specific template. Total cytoplasmic RNA was isolated from cells at various points during two rounds of the cell cycle. For the S1 nuclease protections, 10 μg of total cytoplasmic RNA was hybridized to a 300-nucleotide ^{32}P-labeled template that protects a 150-nucleotide fragment at the 5′ terminus of HSP70 mRNA. The levels of HSP70 mRNA are barely detected in G_1 cells. However, as the cells enter S phase, the level of HSP70 mRNA increases 10- to 15-fold, to a peak coincident with the peak of DNA synthesis at 12 hr and at 36 hr (Fig. 1*C*). The increased levels of HSP70 mRNA detected at the beginning of each round of DNA synthesis are transient, decreasing 80% by the next time point 2 hr later. The results presented here are in contrast with those of Kao *et al.* (28), who observed maximal HSP70 expression during late S/G_2 phase. We cannot provide any explanation for these differences.

HSP70 Synthesis and Levels During the Cell Cycle. To determine whether HSP70 protein synthesis increases as the cytoplasmic levels of HSP70 mRNA increase, cells in G_1, early S, and G_2 were pulse-labeled with [^{35}S]methionine, lysed, and analyzed by two-dimensional gel electrophoresis. HSP70 has a characteristic isoelectric point and molecular mass and is easily distinguished on two-dimensional gels. The levels of HSP70 mRNA were also determined from the same population of cells. Asynchronous cells grown at either 37°C or exposed to a heat shock at 42°C were labeled with [^{35}S]methionine to provide a reference for the location of HSP70 (Fig. 2 *B* and *C*). In cells pulse-labeled in G_1, early S phase, and G_2, high levels of HSP70 synthesis are detected only in S-phase cells (Fig. 2 *D–F*). Thus, the peak of HSP70 protein synthesis during S phase coincides with high levels of cytoplasmic HSP70 mRNA (Fig. 2*A*). Interestingly, HSP70 appears to be the only member of the human 70-kDa heat shock family of proteins that displays this cell cycle-regulated expression.

We followed the accumulation of HSP70 protein during the cell cycle by immunoblot analysis of synchronized cells. Equal numbers of cells at G_1, early and late S, and G_2 were lysed and electrophoretically separated by $NaDodSO_4$/PAGE, and the proteins were transferred to nitrocellulose paper. The filter was incubated with a monospecific antibody (C92) raised against human HSP70 and specific only for HSP70 (27), and the complex was detected with ^{125}I-labeled secondary antibody. The levels of HSP70 are low in G_1 cells, increase 3-fold upon entry into S phase, and decrease gradually thereafter (Fig. 3). We conclude from these studies that both the synthesis and the levels of HSP70 are temporally regulated during the cell cycle.

Subcellular Localization of HSP70 by Indirect Immunofluorescence. The intracellular location of HSP70 was examined by indirect immunofluorescence using the C92 monospecific antibody. Asynchronous populations of HeLa cells grown at physiological temperature (37°C) show diffuse cytoplasmic and nuclear staining with C92 antibody (Fig. 4 *A*

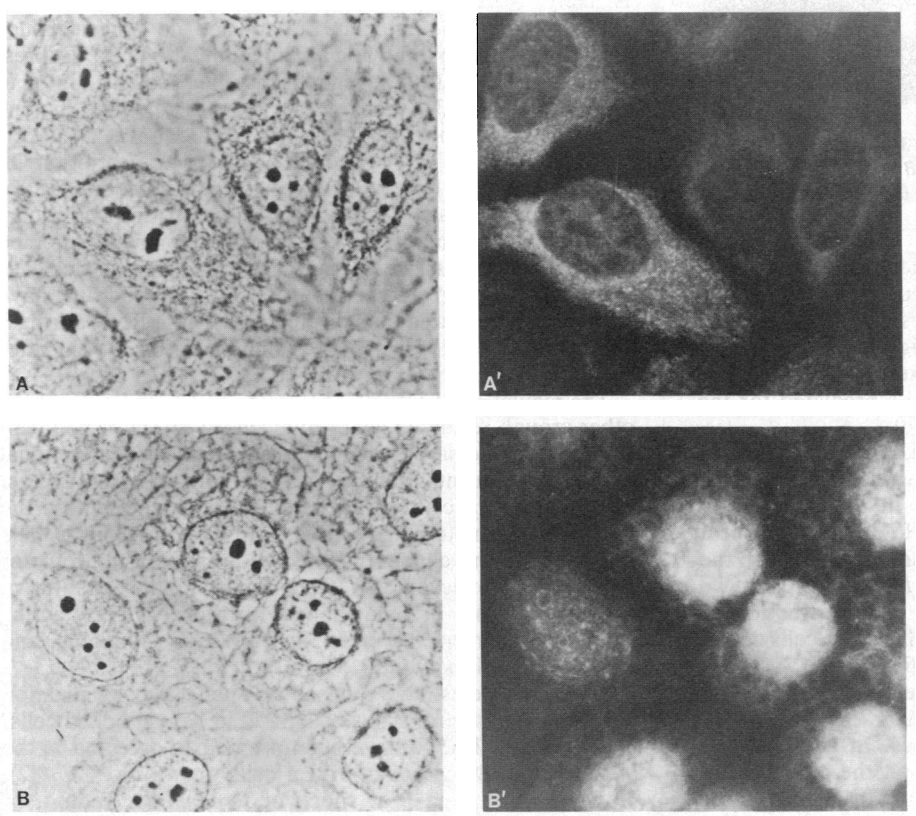


FIG. 4. Subcellular localization of HSP70 in asynchronous populations of HeLa cells at control and heat shock temperatures. Cells growing on glass coverslips were processed for indirect immunofluorescence as described in *Methods*. Phase contrast (*A* and *B*) and fluorescence (*A′* and *B′*) micrographs of asynchronous cells grown at 37°C (*A* and *A′*) and asynchronous cells exposed to 42°C for 60 min (*B* and *B′*).

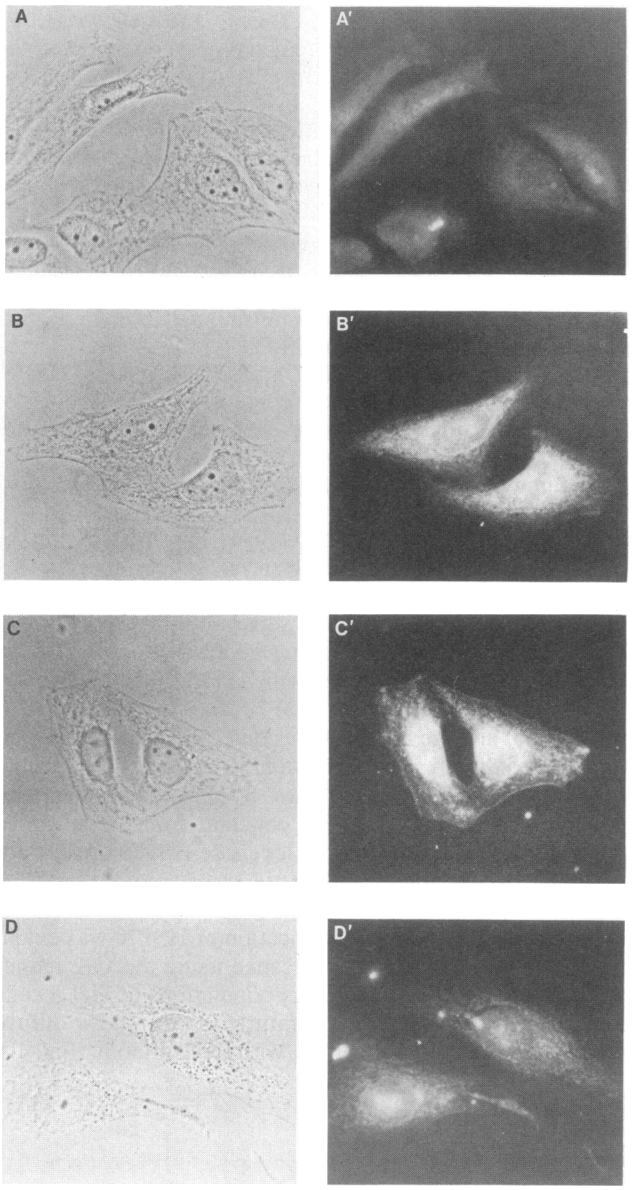


FIG. 5. Subcellular localization of HSP70 in synchronized populations of HeLa cells. (*A* and *A'*) G_1 cells, (*B* and *B'*) G_1/S cells, (*C* and *C'*) S cells, and (*D* and *D'*) G_2 cells. (*A–D*) Phase contrast; (*A'–D'*) immunofluorescence.

and *A'*; refs. 29 and 30). There is a decided heterogeneity in the localization of HSP70 in asynchronous cells at 37°C, with a subset of cells showing a more intense staining pattern than observed for the general population (Fig. 4 *A* and *A'*). This variation in HSP70 distribution observed in asynchronous populations is consistent with the results predicted for the cell cycle-regulated expression of HSP70.

After exposure to a heat shock at 42°C, the localization of HSP70 changes dramatically and has been well characterized (29, 30). There is an overall increase in the intensity of staining due to the induced synthesis of HSP70 following heat shock. HSP70 localizes to the nucleus and, primarily, to the phase-dense nucleolar structures (Fig. 4 *B* and *B'*). Again, we have noticed variability in the levels and distribution of HSP70 in asynchronous heat shocked cells; some cells do not appear to induce HSP70 (Fig. 4 *B* and *B'*).

When synchronized populations of cells are stained with C92 antibody, the pattern of subcellular localization of HSP70 becomes markedly homogeneous. In addition, the distribution of HSP70 changes throughout the cell cycle in cells grown at physiological temperature. In G_1 cells, low levels of HSP70 are distributed diffusely throughout the cytoplasm and the nucleus (Fig. 5 *A* and *A'*). At the G_1/S boundary, the levels of HSP70 increase in both the cytoplasmic and nuclear compartments (Fig. 5 *B* and *B'*). Cells in S phase have high levels of HSP70 primarily localized to the nucleus (Fig. 5 *C* and *C'*). While the amount of HSP70 in the cytoplasm of S phase cells does not appear to decrease during the G_1–S transition, nuclear staining is significantly enhanced. The distribution of HSP70 within the nucleus of S-phase cells is distinct from that of heat shocked cells, where the nucleolar deposition of HSP70 is very prominent. S-phase cells have a more generalized distribution of HSP70 throughout the nucleus, and the nucleolar localization varies (Fig. 5 *C* and *C'*). Later in G_2, HSP70 is dispersed throughout the cell at low levels in both cytoplasmic and nuclear compartments (Fig. 5 *D* and *D'*).

The subcellular localization of HSP70 to the nucleus of S-phase cells reveals that HSP70 is a nuclear-localized antigen during normal conditions of cell growth. Previous studies on HSP70 localization in heat shock or stressed cells have shown a similar pattern of subcellular localization. We conclude from these studies that nuclear compartmentalization is not solely a stress-related phenomenon and must be intrinsic to the HSP70 protein and its cell cycle-regulated expression.

DISCUSSION

In this study, we demonstrate that high levels of HSP70 mRNA accumulate in the cytoplasm of early S-phase cells and then rapidly disappear during mid- to late S and G_2. The rapid increase in HSP70 mRNA in S-phase cells corresponds temporally to an increase in HSP70 protein synthesis that is coincident with its localization to the nucleus of S-phase cells, whereas cells in G_1 and G_2 show a weak generalized cytoplasmic and nuclear distribution of HSP70. Thus, expression of the *HSP70* gene is tightly coupled with the cell cycle of human cells.

The appearance of HSP70 expression with DNA synthesis is likely to be significant, as cells treated with the DNA synthesis inhibitor cytosine arabinoside no longer induce HSP70 expression following serum stimulation (10). Expression of the replication-dependent histones is also coupled to DNA synthesis (31). Transcription rates of these histone genes increase in S phase 2- to 5-fold above G_1 levels (reviewed in ref. 32), while *Hsp70* gene transcription increases 10- to 15-fold following serum stimulation. HSP70 and histones may also share similarities in posttranscriptional regulation. The stability of histone mRNAs may be affected by DNA synthesis rates or perhaps by a specific nuclease involved in mRNA degradation (33, 34). Although we have not directly measured the half-life of HSP70 mRNA during S phase, HSP70 mRNA levels fall rapidly within 2–4 hr after the peak early in S phase (Fig. 1*C*).

The regulation of *HSP70* expression may also be similar to other growth-regulated genes induced by serum or polypeptide growth factors. Recent studies on the transcriptional regulatory elements of c-*fos*, β-interferon, p53, and a yeast histone gene have identified sequence elements within the respective promoters that repress transcription (35–41). The serum regulatory element located within the basal promoter of HSP70 is identical in 9 of 10 positions with the constitutive element and in 6 of 7 positions with the negatively acting element of β-interferon (ref. 38; B. Wu, G. Williams, and R.I.M., unpublished data).

Previous studies have focused on subcellular localization and biochemical properties of HSP70 in heat shocked cells. Low levels of HSP70 have been previously detected in the nucleus and cytoplasm of HeLa cells grown at 37°C (42). However, following heat shock of *Drosophila*, rodent, or

human cells, HSP70 concentrates to the nucleus, and in mammalian cells it is found associated with the nucleolus (42–44). The significance of the nucleolar localization is not clear; however, HSP70 appears to associate with the preribosomal-containing granular region of nucleoli found in stressed cells (27, 45). It has been suggested that one function of HSP70 may be in a protective role during stress and also in cells recovering from stress (27, 45). The results presented in this paper clearly demonstrate that the localization of HSP70 to the nucleus and perhaps to the nucleolus is not entirely dependent on increased temperature or other forms of stress. Rather, the movement of HSP70 into various subcellular compartments at physiological temperature may reflect its association with proteins found in different cellular compartments at various times of the cell cycle. Recently, HSP70 has been shown to form a complex with p53 in rat cells (46). The temporal pattern of growth-regulated expression of p53 is very similar to that described for HSP70 (47). Furthermore, because p53 and HSP70 are both nuclear antigens, an interaction between these proteins may have an important role for the G_1/S-phase transition of the cell cycle.

The temporal pattern of HSP70 expression during S phase, the nuclear localization, induction by trans-activating immortalization proteins, and association with proteins encoded by certain protooncogenes suggests a role for HSP70 during the synthetic phase of the cell cycle. In addition, HSP70 shares 50% amino acid homology with its bacterial analogue dnaK, which is necessary for both DNA and RNA synthesis at restrictive temperatures and is required for bacteriophage λ replication (48–52). Deletion of the equivalent *HSP70* genes in yeast cells results in a temperature-sensitive phenotype for cell growth (53, 54). Although equivalent genetic mutants in mammalian cells are not available to demonstrate the growth-regulating properties of HSP70, we provide circumstantial evidence from the localization of HSP70 to the nucleus of S-phase cells.

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