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Posttranscriptional Regulation of *hsp70* Expression in Human Cells: Effects of Heat Shock, Inhibition of Protein Synthesis, and Adenovirus Infection on Translation and mRNA Stability

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We have examined the posttranscriptional regulation of *hsp70* gene expression in two human cell lines, HeLa and 293 cells, which constitutively express high levels of HSP70. HSP70 mRNA translates with high efficiency in both control and heat-shocked cells. Therefore, heat shock is not required for the efficient translation of HSP70 mRNA. Rather, the main effect of heat shock on translation is to suppress the translatability of non-heat shock mRNAs. Heat shock, however, has a marked effect on the stability of HSP70 mRNA; in non-heat-shocked cells the half-life of HSP70 mRNA is approximately 50 min, and its stability increases at least 10-fold upon heat shock. Moreover, HSP70 mRNA is more stable in cells treated with protein synthesis inhibitors, suggesting that a heat shock-sensitive labile protein regulates its turnover. An additional effect on posttranscriptional regulation of *hsp70* expression can be found in adenovirus-infected cells, in which HSP70 mRNA levels decline precipititously late during infection although *hsp70* transcription continues unabated.

Expression of the major heat shock protein, HSP70, is under complex regulatory controls exerted at both the transcriptional and translational levels (18, 43, 55). Transcription of the *hsp70* gene is transiently induced by environmental stress such as exposure to heat shock or heavy metals; in human cells the *hsp70* gene is also expressed during normal conditions of cell growth at the G1/S boundary of the cell cycle (49) and in response to serum stimulation (71).

The effects of heat shock on posttranscriptional events include the preferential translation of heat shock mRNAs (32, 41, 42, 62). During heat shock, HSP70 mRNA appears to be translated efficiently whereas most preexisting cellular mRNAs (hereafter referred to as "control mRNAs") are poorly translated. The selective translation of heat shock mRNAs is in part due to features within the 5′ noncoding region of the mRNA (15, 34, 46). Although it has been suggested that heat shock mRNAs may require heat shock conditions for their efficient translation (20), there are at least two lines of evidence to suggest that these mRNAs can translate efficiently under non-stress conditions. First, heat shock mRNAs isolated from a variety of organisms translate in extracts prepared from non-heat-shocked wheat germ, *Drosophila*, rabbit reticulocyte, and other animal cells (for example, see references 5, 32, 37, 39, 47, 56, and 62). Second, heat shock mRNAs are transcribed and translated under a variety of normal conditions of cell growth such as the G1/S boundary of the cell cycle (49) and during development and differentiation (9, 10, 59; S. S. Banerji, K. L. Laing, and R. I. Morimoto, Genes Devel., in press).

Other posttranscriptional events induced by heat shock include a block in pre-mRNA processing (72) and effects on mRNA stability (24). During heat shock, HSP70 mRNA appears to be stable relative to its short half-life in cells recovering from heat shock (22, 23). For example, HSP70 mRNA persists in heat-shocked chicken cells well after transcription has returned to preinduced levels and then decays rapidly during recovery at normal growth temperatures (4). These results indicate that the stability of HSP70 mRNA is dependent on the cellular state.

In this study we have examined the stability and translatability of HSP70 mRNA in two human cell lines under conditions of normal cell growth and after heat shock. To accomplish this, we have compared the rates of HSP70 synthesis in control and heat-shocked cells with the relative levels of HSP70 mRNA and its distribution on polyribosomes. Although control mRNAs are efficiently translated in cells maintained at 37°C, they are poorly translated in heat-shocked cells. In contrast, HSP70 mRNA is translated efficiently at both control and heat shock conditions. We also demonstrate that HSP70 mRNA is short-lived in non-heat-shocked cells, decaying with a half-life of approximately 50 min. The stability of HSP70 mRNA increases during heat shock or in the presence of protein synthesis inhibitors. During adenovirus infection, HSP70 mRNA levels decline to undetectable levels while transcription of the *hsp70* gene continues.

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MATERIALS AND METHODS

Plasmids, cell culture, and conditions of heat shock and adenovirus infection. Plasmid pH2.3 (69) contains the protein-coding region of a human *hsp70* gene. Plasmid pHFβA-1 (29) contains a human β-actin cDNA. Plasmid pSpIVS1 (Y.-D. Choi, Ph.D. dissertation, Northwestern Univ., 1986) contains map units 69.5 to 72.8 of the adenovirus 5 genome and hybridizes to the L4 mRNA encoding the 100,000-molecular-weight (100K) protein. Plasmid pJOLC3 (67) contains map units 0 to 7.5 of the adenovirus 5 genome and hybridizes to the E1A and E1B mRNAs.

HeLa (JW36) cells were maintained in spinner culture at 0.5×10^5 to 5.0×10^5 cells per ml in Joklik minimal essential medium containing 5% fetal calf serum. Fresh medium was added to the cultures 20 to 30 h before each experiment. Heat shock was effected by suspending cells in conditioned medium prewarmed to the heat shock temperature (42 to 43°C) and continuing the incubation at the heat shock temperature for up to 2 h.

Cell line 293 (27), a human embryonic kidney cell line transformed by the pre-early regions of adenovirus 5, was grown in 100-mm dishes in Dulbecco modified Eagle medium containing 5% calf serum and heat shocked as described. Infections of cell line 293 cells with adenovirus 5 (wild type strain *dl*704 [12], a generous gift of B. Thimmappaya) were performed as described (70).

Metabolic labeling with [^{35}S]methionine. A 1-ml sample containing 10^6 cells was removed, collected by centrifugation, washed with methionine-free medium, suspended in 0.1 ml of methionine-free medium containing 50 μCi of [^{35}S]methionine per ml, and incubated at 37°C for 15 min. The cells were washed in phosphate-buffered saline, collected, and frozen. The cell pellets were lysed in gel sample buffer (62.5 mM Tris hydrochloride, pH 6.8, 2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue), sonicated, and boiled. The labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (13, 40) and fluorography (16). Incorporation of [^{35}S]methionine into proteins was determined by precipitation with trichloroacetic acid after base hydrolysis of charged tRNAs. The precipitates were collected on glass-fiber filters and analyzed by liquid scintillation counting.

RNA purification and analysis and transcription in isolated nuclei. For RNA purification, a sample of 1.5×10^7 cells was removed, washed in phosphate-buffered saline, pelleted, and frozen. The cell pellets were lysed in 0.5 ml of 0.1 M NaCl–10 mM Tris hydrochloride (pH 8)–2 mM EDTA–1% Nonidet P-40–0.5% sodium deoxycholate–1% 2-mercaptoethanol. The cell lysate was homogenized by passage through a 27 gauge needle, and the nuclei were pelleted by centrifugation at 12,000 × *g*. The supernatant was removed, adjusted to 0.5% SDS, digested with 200 μg of Proteinase K per ml, extracted with phenol-chloroform, precipitated with ethanol, and suspended in sterile distilled water.

In vitro translation reactions were primed with 5 μg of total cytoplasmic RNA added to an mRNA-dependent micrococcal nuclease-treated (51) rabbit reticulocyte lysate (Green Hectars, Oregon, Wis.). The translation assays were performed as described (5).

For Northern (RNA) blot analysis, 5 μg of total cytoplasmic RNA was denatured with glyoxal and dimethyl sulfoxide (48), electrophoretically separated on 1.2% agarose gels containing 10 mM sodium phosphate buffer (pH 7), and blotted to nitrocellulose (64). The filters were baked, hybridized to ^{32}P-labeled plasmids, washed at 65°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) containing 0.2% SDS, followed by 0.2× SSC at 65°C, and exposed to X-ray film with an intensifying screen.

Nuclease S1 protection assays (11) were performed essentially as described (69). Total cytoplasmic RNA was hybridized to ^{32}P-labeled DNA probes; the RNA-DNA hybrids were digested with S1 nuclease (Boehringer Mannheim, Indianapolis) and electrophoretically separated on 4% acrylamide gels, containing 8 M urea, or on alkaline agarose gels. HSP70 RNA was assayed using 5′- or 3′-specific probes. For 5′ end analysis, DNA was labeled at the *Bam*HI site (position +150 relative to the transcription initiation site of the human *hsp70* gene) using T4 polynucleotide kinase; for 3′ end analysis, the DNA was labeled at the same site using the Klenow fragment of *Escherichia coli* DNA polymerase I. The 5′ probe protects a 150-nucleotide fragment, and the 3′ probe protects a 2.15-kilobase fragment (69). The β-actin probe was prepared by digestion of the plasmid pHFβA-1 (29) with *Bst*EII and labeling with T4 polynucleotide kinase. This probe protects a fragment of 504 nucleotides. Labeling of the 5′ and 3′ ends was performed using published procedures (45).

Transcription in isolated nuclei (26) and subsequent isolation and hybridization of ^{32}P-labeled "run-on" transcripts were performed essentially as described (5).

Analysis of mRNA distribution on polysomes. For polysome analysis, a sample of 1.5×10^7 cells was removed; cycloheximide was added to 100 μg/ml, and the cells were immediately quick-chilled by immersion in a dry ice-methanol bath for 5 s and ice water for 30 s with constant agitation. The cells were pelleted, washed with ice-cold phosphate-buffered saline containing 100 μg of cycloheximide per ml, pelleted, and frozen on dry ice-methanol. The cell pellets were lysed by the addition of 0.5 ml of polysome buffer (0.3 M KCl, 5 mM $MgCl_2$, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.4) containing 0.5% Nonidet P-40, 100 μg of cycloheximide per ml, and 5 mM dithiothreitol. The lysate was homogenized by passage through a 27 gauge needle and centrifuged at 12,000 × *g* for 5 min to remove nuclei and cell debris. A sample of 200 μl was layered on a 12-ml 15 to 45% sucrose gradient in polysome buffer and centrifuged for 60 min at 38,000 rpm in a Beckman SW41 rotor. The gradients were monitored by A_{260} and fractionated by pumping from the top; 24 fractions of 0.5 ml were collected. Hybridization analysis of fractions was performed on formaldehyde-denatured samples (38). Portions of each gradient fraction were added to an equal volume of 2× NaPF (1× NaPF is 1 M NaCl, 40 mM sodium phosphate buffer, pH 7, and 6% formaldehyde), heated to 65°C for 5 min, and cooled to room temperature. The samples were centrifuged to remove any insoluble material and applied to nitrocellulose by using a Schleicher & Schuell Minifold apparatus. Hybridization to radiolabeled DNA fragments was as described.

RESULTS

HSP70 synthesis and mRNA levels in control and heat-shocked cells. We assessed the translation of HSP70 mRNA in two human cell lines, HeLa and 293, under conditions of normal cell growth or after heat shock. The level of HSP70 synthesis was measured in HeLa cells maintained at 37°C or heat shocked at 42 and 43°C by pulse-labeling with [^{35}S]methionine and analysis on one-dimensional SDS-PAGE. HeLa cells grown at 37°C constitutively synthesize a low level of HSP70. Heat shock causes a rapid change in the pattern of protein synthesis, most notably the increased synthesis of the major heat shock protein HSP70 and the slightly elevated synthesis of HSP89 (Fig. 1). During a 2-h 42°C heat shock, HSP70 synthesis increased 20- to 30-fold, whereas incubation at 43°C resulted in a 15- to 20-fold induction (Table 1). Incubation at 42°C for up to 2 h had little effect on overall levels of protein synthesis; for example, actin synthesis was reduced by less than 10% (Table 1). This is in contrast to incubation at 43°C, which suppressed overall levels of protein synthesis; for example, actin synthesis was reduced by 75%.

To determine whether changes in the level of HSP70 and actin synthesis in cells shocked at 42 or 43°C corresponded to equivalent changes in the corresponding mRNAs, we isolated total cytoplasmic RNA from each sample shown in Fig. 1A for translation in nuclease-treated rabbit reticulocyte lysates. We then compared the in vivo levels of HSP70 synthesis with those obtained in vitro to determine whether HSP70 mRNA might be preferentially translated in heat-

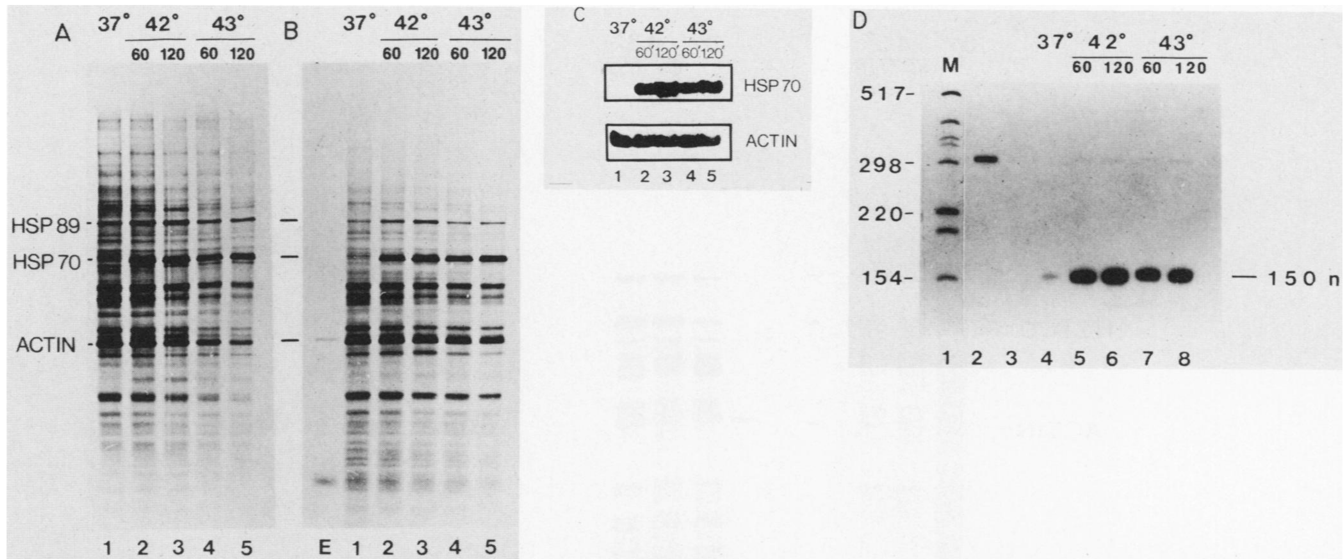

FIG. 1. Effects of heat shock on protein synthesis and mRNA abundance in HeLa cells. (A) Protein synthesis in vivo. HeLa cells were incubated at 37°C for 1 h or heat shocked at 42 or 43°C for the indicated times (minutes). A sample of cells was pulse-labeled with [^{35}S]methionine, and the labeled proteins were analyzed by SDS-PAGE and fluorography. Lane 1, Proteins synthesized in cells incubated at 37°C. Lanes 2 and 3, Proteins synthesized by cells incubated at 42°C for 60 and 120 min, respectively. Lanes 4 and 5, Proteins synthesized by cells incubated at 43°C for 60 and 120 min, respectively. The positions of HSP70, HSP89, and actin are indicated. (B) Protein synthesis in vitro. HeLa cells were incubated as described above; total cytoplasmic RNA was isolated and used to program protein synthesis in a nuclease-treated rabbit reticulocyte extract. The translation products were analyzed by SDS-PAGE and fluorography. Lane E, Endogenous protein synthesis of the lysate in the absence of added RNA. Lanes 1 through 5, Translation products directed by RNA isolated from cells incubated as described for lanes 1 through 5 in panel A. (C) Northern blot analysis. RNA isolated from cells incubated as described above was denatured with glyoxal and dimethyl sulfoxide and separated on a neutral agarose gel; the RNA was then blotted to nitrocellulose and probed with HSP70 and β-actin sequences as described in the text. Lanes 1 through 5, RNA isolated from cells incubated as described for lanes 1 through 5 in panel A. (D) Nuclease S1 analysis. RNA isolated from cells incubated as described above was hybridized to a 300-base-pair probe derived from the 5′ end of the human *hsp70* gene. The probe was ^{32}P labeled at a *Bam*HI site at position +150 (relative to the transcription initiation site) and extends to position −150. The RNA-DNA hybrids were digested with S1 nuclease, and the protected fragments were visualized by electrophoresis and autoradiography. Lane 1, Marker DNA fragments; sizes (on the left) are given in nucleotides. Lane 2, Undigested template DNA. Lane 3, DNA fragments protected after hybridization to *E. coli* tRNA. Lanes 4 through 8, DNA fragments protected after hybridization to RNA isolated from cells incubated as described for lanes 1 through 5 in panel A.

shocked cells. Changes in HSP70 synthesis were compared with those of a representative control protein, actin. The fluorograms of the respective SDS-PAGE gels (Fig. 1B) were quantified, and the results are shown in Table 1. The increased synthesis of HSP70, as measured by [^{35}S]methionine pulse-labeling, was proportional to the increase in HSP70 mRNA inferred by analysis of the in vitro translation results. In contrast, actin mRNA levels assayed by in vitro translation remained relatively constant, although actin synthesis was suppressed at 43°C.

We confirmed the in vitro translation results by directly measuring HSP70 and β-actin mRNA levels by Northern blot analysis (Fig. 1C). The relative levels of actin and HSP70 mRNAs assayed by Northern blot were similar to the levels obtained by in vitro translation (Table 1). We also examined the levels of HSP70 mRNA originating from the

TABLE 1. Effects of heat shock on protein synthesis and mRNA levels in HeLa cells[a]

Heat shock		Protein synthesis[b]			mRNA levels[c]				
					Actin		HSP70		
Temp (°C)	Time (min)	Total	Actin	HSP70	In vitro translation	Northern blot	In vitro translation	Northern blot	S1 nuclease
37	60	91	100	3	100	100	3	3.8	3.6
42	60	100	96	82	93	88	59	69	59
42	120	91	93	100	84	81	100	100	100
43	60	68	56	53	98	93	61	65	39
43	120	49	28	72	89	76	89	77	59

[a] The values listed in the table are given as the percent of maximum value.

[b] Total protein synthesis was determined by precipitation of ^{35}S-labeled protein by trichloroacetic acid and scintillation counting. Actin and HSP70 syntheses were determined by scanning densitometry of the appropriate bands of the fluorogram shown in Fig. 1A.

[c] RNA levels for β-actin and HSP70 were determined as follows. In vitro translation: RNA was used to program protein synthesis in an in vitro translation system; the level of synthesis was determined by scanning densitometry of the appropriate bands of the fluorogram shown in Fig. 1B. Northern blot: RNA was analyzed by Northern blot (Fig. 1C), and the level of hybridization was determined by scanning densitometry. S1 nuclease: The HSP70 mRNA levels were determined by S1 nuclease analysis (Fig. 1D).

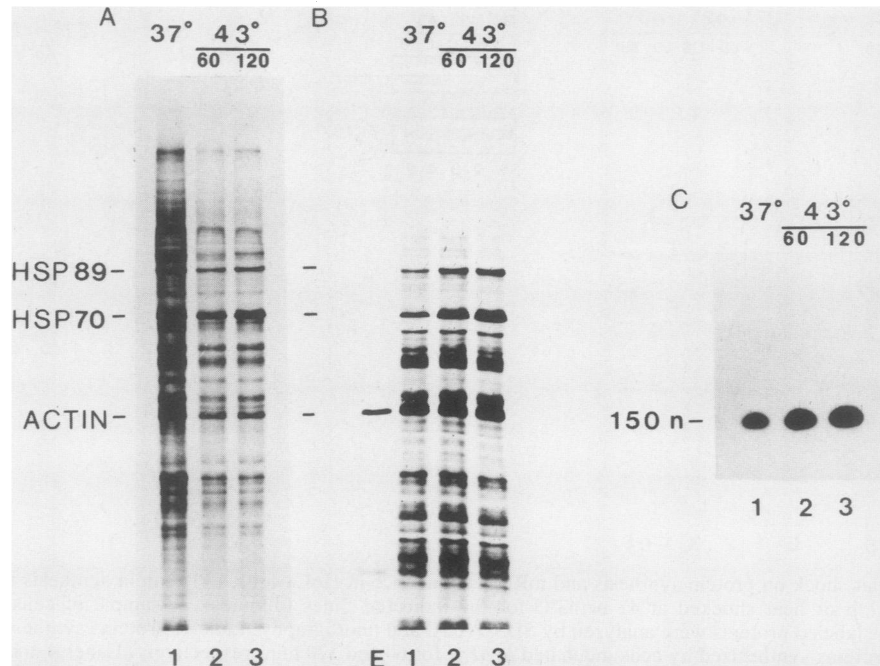

FIG. 2. Effects of heat shock on protein synthesis and mRNA levels in 293 cells. (A) Protein synthesis in vivo. Cell line 293 cells were maintained at 37°C or heat shocked at 43°C for 60 or 120 min. The cells were pulse-labeled with [^{35}S]methionine, and the labeled proteins were analyzed by SDS-PAGE and fluorography. Lane 1, Proteins synthesized by cells incubated at 37°C. Lanes 2 and 3, Proteins synthesized by cells incubated at 43°C for 60 or 120 min, respectively. (B) Protein synthesis in vitro. RNA was isolated from cells incubated as described above and translated in vitro as described for Fig. 1. Lane E, Endogenous protein synthesis of the lysate in the absence of added RNA. Lanes 1 through 3, Translation products directed by RNA isolated from cells incubated as described for lanes 1 through 3 in panel A. (C) HSP70 mRNA levels. RNA was isolated from cells incubated as described above and assayed by S1 nuclease protection for the presence of HSP70 mRNA as described for Fig. 1D. HSP70 mRNA levels in cells incubated at 37°C (lane 1) and at 43°C for 60 (lane 2) and 120 (lane 3) min are shown.

cloned HSP70 gene, using S1 nuclease analysis to minimize possible complexities arising from multiple *hsp70* genes in the human genome (17, 30, 66, 69). The results obtained by S1 nuclease protection were identical to those obtained by Northern blot analysis and in vitro translation assays (Fig. 1D, Table 1). We conclude that HSP70 synthesis is not perturbed by heat shock, whereas the translation of control mRNAs such as actin is suppressed by a 43°C heat shock.

Direct evidence that translation of HSP70 mRNA does not require heat shock-induced alterations in the protein synthetic machinery was obtained by a quantitative analysis of protein synthesis and mRNA levels in 293 cells, which constitutively synthesize high levels of HSP70 (36, 50, 69). We compared the in vivo levels of HSP70 synthesis with the levels of HSP70 mRNA in control and heat-shocked 293 cells. Quantification of in vivo-labeled proteins analyzed by SDS-PAGE revealed that HSP70 is constitutively synthesized and its synthesis increases after heat shock (Fig. 2A). We compared the level of HSP70 synthesis with the level of HSP70 mRNA in these cells (Fig. 2B and C) and found that the amount of HSP70 mRNA increases with heat shock and is proportional to the increase in HSP70 synthesis (Table 2). These results indicate that HSP70 mRNA in 293 cells is translated with similar efficiency in control and heat-shocked cells. Therefore, heat shock is not an essential condition for the translation of HSP70 mRNA.

Distribution of HSP70 mRNA on polysomes. We assessed the functional state of HSP70 and actin mRNAs in HeLa and 293 cells on the basis of their distribution on polysomes (Fig. 3 and 4). This approach offers an independent means of demonstrating that the HSP70 mRNA is translationally active. Control or heat-shocked HeLa and 293 cells were lysed; a postnuclear supernatant was prepared and subjected to sedimentation on 15 to 45% sucrose gradients. The absorbance profiles (A_{260}) of polysomes from HeLa and 293 cells treated at control or heat shock temperatures revealed that incubation at 42°C has little effect on protein synthesis, whereas a 43°C heat shock causes polysome disaggregation and an increase in free ribosomes (Fig. 3A), concomitant with the reduction in overall protein synthesis (Table 1). Portions from the fractionated sucrose gradients were denatured with formaldehyde, applied to nitrocellulose, and hybridized to a ^{32}P-labeled plasmid containing the human

TABLE 2. Effects of heat shock on HSP70 synthesis and mRNA levels in 293 cells[a]

Heat shock: Temp (°C)	Heat shock: Time (min)	HSP70 synthesis[b]	HSP70 mRNA (in vitro)[c]	HSP70 mRNA (S1)[d]
37		37	30	23
43	60	53	68	73
43	120	100	100	100

[a] The values listed in the table are given as the percent of maximum value.

[b] HSP70 synthesis in vivo was determined by scanning densitometry of the appropriate band of the fluorogram shown in Fig. 2A.

[c] HSP70 mRNA levels were determined by in vitro translation and scanning densitometry of the fluorogram shown in Fig. 2B.

[d] HSP70 mRNA levels were determined by S1 nuclease analysis and scanning densitometry of the autoradiogram shown in Fig. 2C.

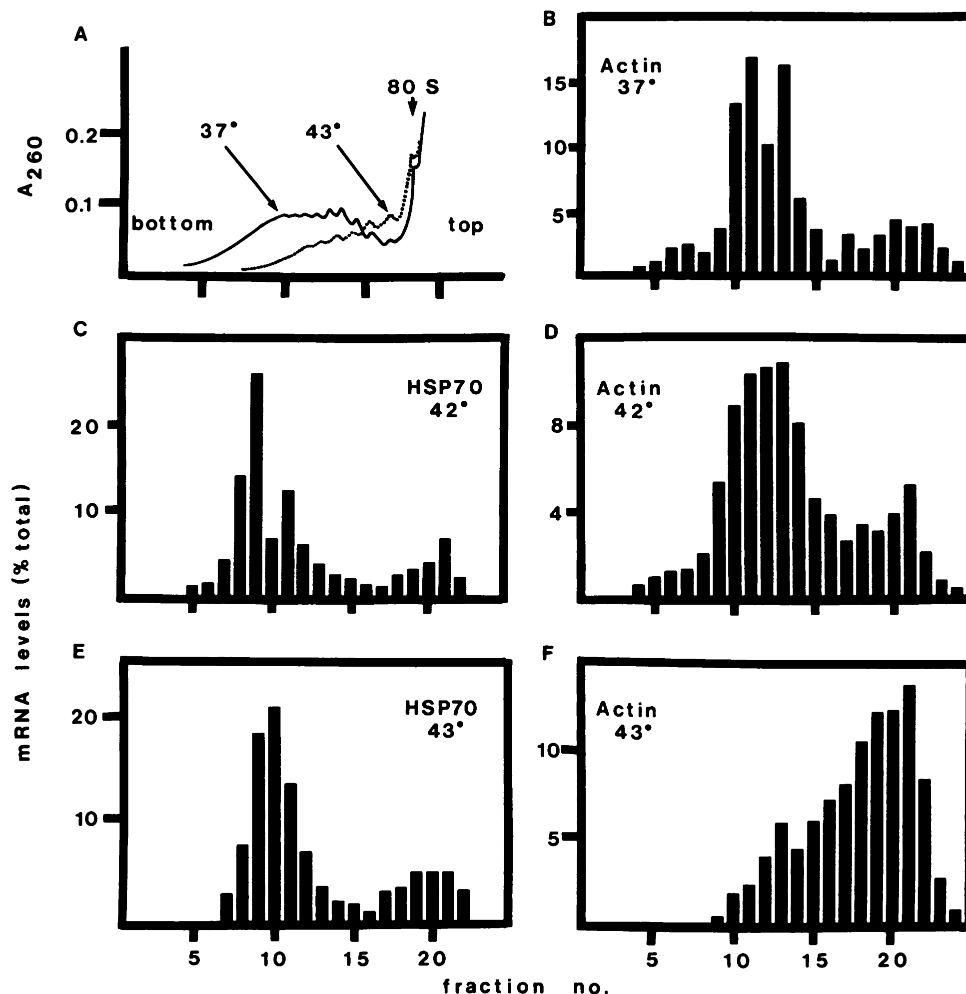

FIG. 3. Polysome distribution of HSP70 and β-actin mRNAs in HeLa cells. Cells were incubated as described; a postnuclear supernatant was prepared and sedimented in sucrose gradients as described in the text. Gradient fractions (24 per gradient) were denatured with formaldehyde, applied to nitrocellulose, and hybridized to HSP70 and β-actin gene probes. The levels of hybridization were determined by scanning densitometry. (A) Absorbance profiles from sucrose gradients. The direction of sedimentation is from right to left. The position of the 80S monosome peak is indicated. The absorbance profiles of polysomes from cells incubated at 37°C (——) and 43°C (. . . .) are shown; the absorbance profile of polysomes from cells incubated at 42°C (not shown) is similar to that of polysomes from 37°C-treated cells. (C and E) HSP70 mRNA distribution in cells incubated at 42°C for 60 min or at 43°C for 120 min, respectively. The units given on the ordinate are the percent of total hybridization. (B, D, and F) Actin mRNA distribution in cells incubated at 37°C, at 42°C for 120 min, or at 43°C for 60 min.

hsp70 gene (pH2.3) and, separately, to the human β-actin gene probe (pHFβA-1). The intensity of the hybridization signal was quantified by scanning densitometry and normalized to internal standards on each filter.

The distribution of HSP70 mRNA on polysomes from heat-shocked HeLa cells and from control and heat-shocked 293 cells is shown in Fig. 3 and 4. The peak of HSP70 mRNA corresponds to fractions 8 through 11, which correspond to 10 to 20 ribosomes per mRNA (Fig. 3C and E; Fig. 4B and C). In HeLa cells we found that 77 to 80% of the HSP70 mRNA is polysome associated and that over 85% is polysome associated in either control or heat shock-treated 293 cells. The similarity in distribution of HSP70 mRNA on polysomes of control 293 cells to that of heat-shocked 293 or heat-shocked HeLa cells indicates that HSP70 mRNA is efficiently translated in 293 cells maintained at 37°C.

The effects of heat shock on actin mRNA distribution on polysomes of HeLa cells were as predicted from the comparisons of in vivo and in vitro translation; approximately 70% of actin mRNA was associated with 5 to 10 ribosomes (fractions 10 through 14) in control (Fig. 3B) or 42°C heat-shocked cells (Fig. 3D), whereas treatment at 43°C for 60 min, which results in a 50% reduction in actin synthesis, resulted in a 50% reduction in polysome-associated actin mRNA (Fig. 3F). These results are consistent with a heat shock-induced block at the level of translational initiation of actin mRNA.

HSP70 mRNA is short-lived under conditions of normal cell growth. We estimated the half-life of HSP70 mRNA by arresting transcription with actinomycin D and following HSP70 synthesis and HSP70 mRNA levels. A concentration of 1.0 μg of actinomycin D per ml, sufficient to completely inhibit heat-induced transcription of HSP70, was added to cultures of 293 cells. After 2 h at 37 or 43°C, the cells were pulse-labeled with [^{35}S]methionine and analyzed by SDS-PAGE. The level of HSP70 synthesis in cells treated with

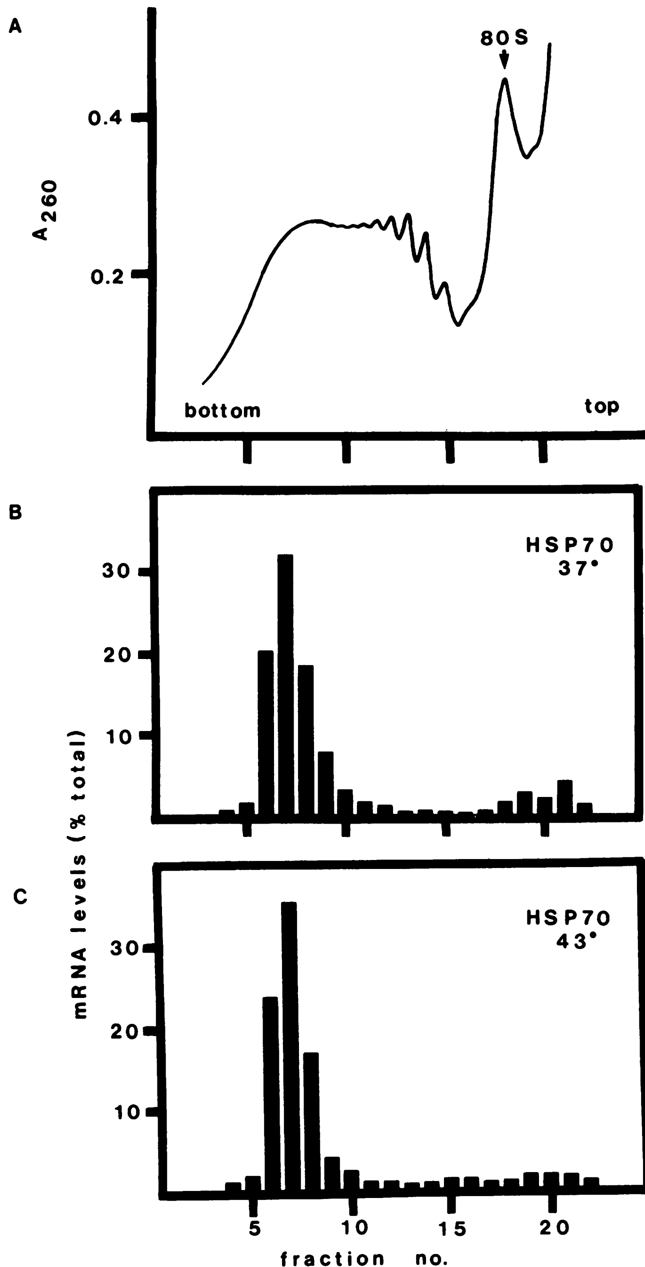

FIG. 4. Polysome distribution of HSP70 and mRNA in 293 cells. Analyses were performed as described for Fig. 3. (A) Absorbance profiles of polysomes from cells incubated at 37°C. (B and C) HSP70 mRNA distribution on polysomes from cells incubated at 37 or 43°C, respectively.

actinomycin D was severely reduced relative to the prominent HSP70 synthesis in the untreated cells (Fig. 5A). These results suggest either that HSP70 protein synthesis requires continued transcription or that HSP70 mRNA is short-lived. To distinguish between these possibilities, we measured the level of HSP70 mRNA in cells incubated in the presence or absence of actinomycin D. Total cytoplasmic RNA was isolated and used for S1 nuclease protection using a ^{32}P-labeled template containing the 5′ end of the human *hsp70* gene. In 293 cells incubated with actinomycin D for 2 h at 37°C, the level of HSP70 mRNA decreased 10-fold (Fig. 5B), equivalent to the reduction in HSP70 protein synthesis. Therefore, HSP70 mRNA appears to have a half-life of significantly less than 2 h. When actinomycin D-treated cells were incubated at 43°C for 2 h, approximately 50% of the HSP70 mRNA remained, suggesting that heat shock stabilizes this mRNA (Fig. 5B).

A more accurate estimate of the half-life of HSP70 mRNA was obtained by removing samples of actinomycin D-treated cells at various times up to 4 h and isolating total cytoplasmic RNA for S1 nuclease analysis using 5′ and 3′ end-labeled hybridization probes (Fig. 6A and B). With either hybridization probe, a half-life of 50 min was obtained (Fig. 6C).

An independent measure of HSP70 mRNA half-life was obtained by determining the amount of time required for HSP70 mRNA to attain a steady-state level of [^{3}H]uridine incorporation (28). Cultures of 293 cells were incubated with [^{3}H]uridine; samples were removed at various time points, and total cytoplasmic RNA was isolated for hybridization to denatured DNA of the *hsp70* and β-actin genes immobilized on nitrocellulose filters. Whereas ^{3}H-labeled β-actin mRNA continued to accumulate throughout the 5-h labeling period, ^{3}H-labeled HSP70 mRNA levels reached saturation in 1 to 2 h (data not shown). These results indicate that HSP70 mRNA is short-lived, in agreement with the half-life obtained using actinomycin D.

Heat shock or arrest in protein synthesis increases the stability of HSP70 mRNA. We examined whether heat shock affected the stability of HSP70 mRNA in 293 cells. Samples of cells were incubated at 37 or 43°C for 30 min. Actinomycin D was added to 1.0 μg/ml, and the cells were further incubated at 37 or 43°C. HSP70 mRNA levels were determined by S1 nuclease analysis. In contrast to the short half-life for HSP70 mRNA obtained in cells at 37°C, cells incubated at heat shock temperatures in the presence of actinomycin D had significantly higher levels of HSP70 mRNA (Fig. 7). Furthermore, HSP70 mRNA showed in-

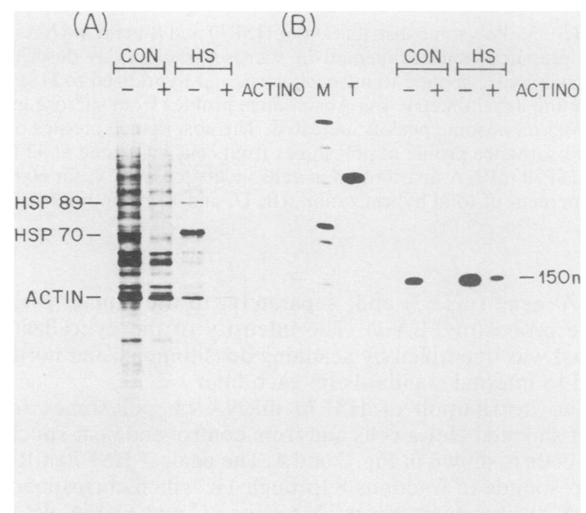

FIG. 5. Effect of actinomycin on HSP70 synthesis and mRNA levels in 293 cells. (A) Protein synthesis. Cells were incubated in the absence (−) or presence (+) of 1 μg of actinomycin D (ACTINO) per ml at 37°C (CON) or 43°C (HS) for 2 h. Protein synthesis was analyzed by pulse-labeling and SDS-PAGE as for Fig. 1. (B) HSP70 mRNA levels. Cells were incubated as described above; RNA was purified, and HSP70 mRNA levels were determined by S1 nuclease analysis as described for Fig. 1. M, Marker DNA fragments; T, undigested template DNA.

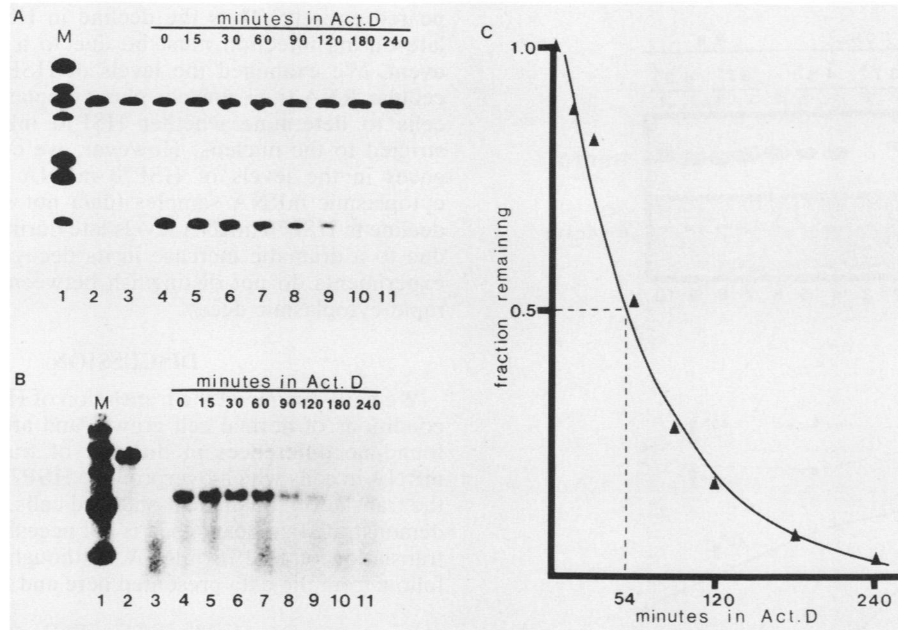

FIG. 6. HSP70 mRNA decay in transcriptionally arrested 293 cells. Actinomycin D was added at 1 μg/ml to cultures of 293 cells. At the indicated times, cells were harvested and RNA was isolated. (A) S1 nuclease analysis of the 5′ end of HSP70 mRNA. RNA isolated from actinomycin-treated cells was hybridized to ^{32}P-labeled DNA probe that spans the 5′ end of the human *hsp70* gene. The DNA-RNA hybrids were digested with S1 nuclease and analyzed as described for Fig. 1. The probe protects the first 150 nucleotides of the mRNA. Lane 1, Marker DNA fragments. Lane 2, Undigested template DNA. Lane 3, Protected fragments from DNA hybridized to *E. coli* tRNA. Lanes 4 through 11, DNA fragments protected by RNA isolated from 293 cells incubated in actinomycin D for the indicated times. (B) S1 nuclease analysis of the 3′ end of HSP70 mRNA. The probe used was ^{32}P labeled at the 3′ end of the *Bam*HI site at position +150 of the human *hsp70* gene and protects the 3′-most 2.15 kilobases of the mRNA. The hybrids were digested with S1 nuclease as described and analyzed by alkaline agarose gel electrophoresis. Lane 1, Marker DNA fragments. Lane 2, Undigested template DNA. Lane 3, Protected fragments from DNA hybridized to *E. coli* tRNA. Lanes 4 through 11, DNA fragments protected by RNA isolated from cells incubated in actinomycin D for the indicated times. (C) Half-life of HSP70 mRNA. HSP70 mRNA levels in actinomycin-treated 293 cells were quantified by scanning densitometry of the autoradiogram shown in panel A. A first-order exponential decay curve was fitted to the data by using a least-squares method. The time at which 50% of HSP70 mRNA has decayed is indicated by the dotted line.

creased stability in heat-shocked cells whether the cells were heat shocked for 30 min before actinomycin D addition or heat shocked after actinomycin D addition. These results suggest that heat shock suppresses the activity or level of an mRNA stability factor.

Expression of the *hsp70* gene during the cell cycle and the short half-life of HSP70 mRNA are reminiscent of other growth-regulated gene products such as histones, interferon, and the product of *myc* (19, 21, 31, 44, 53, 60, 61). Another feature common to this class of gene products is an increase in mRNA stability after an arrest in protein synthesis. We examined whether the stability of HSP70 mRNA increased in cells incubated in the presence of the protein synthesis inhibitors cycloheximide, which blocks elongation, and pactamycin, which affects initiation (reviewed in reference 65). Cultures of 293 cells were left untreated, incubated with 100 μg of cycloheximide per ml or 0.1 μM pactamycin, or heat shocked at 43°C for 30 min (the concentrations of these inhibitors were sufficient to inhibit greater than 98% of incorporation of [^{35}S]methionine into protein; data not shown). The cells were then treated with actinomycin D for 0 or 2 h, and the levels of HSP70 mRNA were determined by S1 nuclease analysis. The results showed that stability of HSP70 mRNA increased when protein synthesis was inhibited (Fig. 8).

Modulation of HSP70 mRNA levels in adenovirus-infected cells. Another form of posttranscriptional regulation occurs during adenovirus infection. Although host transcription remains unperturbed, transport of host cell mRNAs to the cytoplasm and translation of host mRNAs are repressed late during infection (1, 7). These effects appear to mediated at least in part by the adenovirus E1B-55K gene product (2, 52). We examined whether HSP70 might be similarly affected, as *hsp70* has been shown to be among the few genes that are induced during adenovirus infection (36, 50, 70).

We analyzed the effects of adenovirus infection on *hsp70* transcription and mRNA levels in 293 cells to determine whether adenovirus affects HSP70 mRNA stability in cells that constitutively express *hsp70*. During infection of 293 cells with adenovirus type 5, the synthesis of most cellular proteins, including HSP70, did not decrease through 12 h postinfection (p.i.); however, after 16 h p.i. the synthesis of HSP70 and actin, like most other cellular proteins, was barely detected (Fig. 9). The expression of adenovirus-encoded proteins (II, 100K, III, and IV), first detected at 12 h, increased throughout infection. We examined whether changes in in vivo protein synthesis reflected mRNA levels by isolating total cytoplasmic RNA for Northern blot or S1 nuclease analysis. HSP70 mRNA levels increased during infection (between 4 and 8 h p.i.) and abruptly disappeared between 12 and 16 h (Fig. 10A), parallel with the transient induction and repression of HSP70 protein synthesis. In contrast, the level of actin mRNA remained unchanged throughout the same period of adenovirus infection (Fig. 10B), and L4-100K mRNA, which was first detected at 12 h, continued to increase through 16 h (Fig. 10C). We conclude

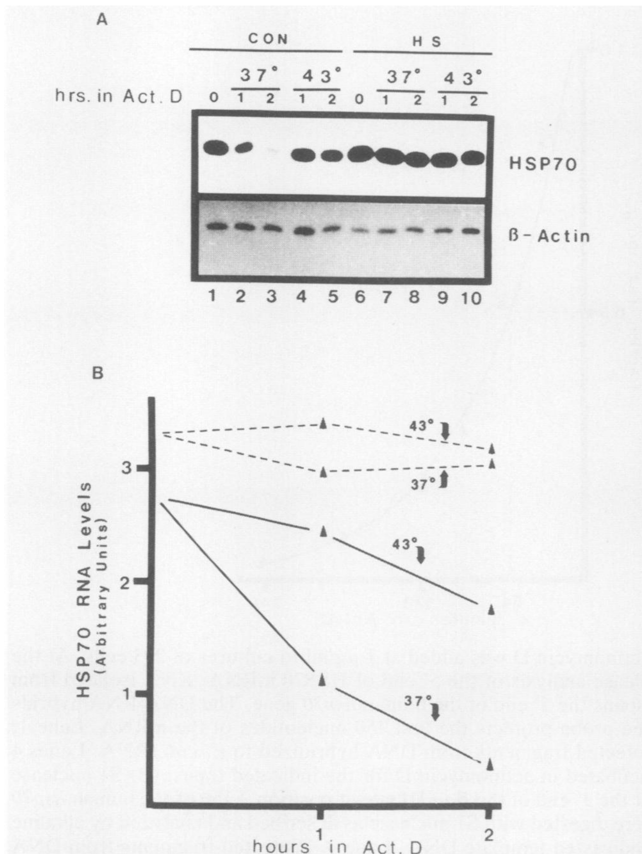

FIG. 7. Effects of heat shock on mRNA stability. Cultures of 293 cells were incubated at 37°C (CON) or at 43°C (HS) for 30 min. A sample of cells was removed for the zero time point. For the remaining cells, actinomycin D was added to 1 μg/ml and the cells were further incubated at 37 or 43°C for 1 or 2 h. RNA was isolated, and the levels of HSP70 and β-actin mRNA were determined by S1 nuclease analysis. (A) S1 nuclease protection using the HSP70 or β-actin gene probes. Shown are RNA levels in cells incubated at 37°C (lanes 1 through 5) or 43°C (lanes 6 through 10) before the addition of actinomycin. (B) HSP70 mRNA decay in control and heat-shocked cells. HSP70 mRNA levels were quantified by scanning densitometry of the autoradiogram shown in panel A. Solid lines, HSP70 mRNA decay in cells incubated at 37°C before actinomycin treatment. Dashed lines, HSP70 mRNA decay in cells incubated at 43°C before actinomycin treatment.

that the mechanism of host repression, as characterized by the reduction in actin synthesis but not in actin mRNA levels, as observed by other laboratories for numerous cellular mRNAs (1, 6, 7), may not extend to *hsp70*.

We examined the effect of adenovirus infection on *hsp70* transcription to determine whether the lack of HSP70 mRNA late during infection was a consequence of decreased transcription. Accordingly, nuclei were isolated from 293 cells infected for various times with adenovirus for in vitro "run-on" transcriptions to assess the relative rate of *hsp70* transcription. As expected, the transcription of the L4 mRNA was not detected in uninfected cells and increased rapidly between 12 and 16 h p.i. (Fig. 11). Transcription of the E1A/E1B genes is detected in uninfected 293 cells (27) and increases during infection. We found that neither β-actin gene transcription nor *hsp70* transcription was reduced by more than twofold during the time course of adenovirus infection (Fig. 11), although HSP70 mRNA levels disappeared entirely. Thus the decline in HSP70 mRNA levels late during infection must be due to a posttranscriptional event. We examined the levels of HSP70 mRNA in total cellular RNA (i.e., nuclear plus cytoplasmic) from infected cells to determine whether HSP70 mRNA might be restricted to the nucleus. However, we could find no differences in the levels of HSP70 mRNA between total and cytoplasmic mRNA samples (data not shown). Hence the decline in HSP70 mRNA levels late during infection must be due to a dramatic increase in its decay rate, although our experiments do not distinguish between rapid nuclear and rapid cytoplasmic decay.

DISCUSSION

We have examined the translation of HSP70 mRNA under conditions of normal cell growth and after heat shock. We found no differences in the rate of translation of HSP70 mRNA in cells which overproduce HSP70 at 37°C relative to the translation rate in heat-shocked cells. Our results clearly demonstrate that heat shock is not necessary for the efficient translation of HSP70 mRNA. Although these conclusions follow from the data presented here and from the findings of

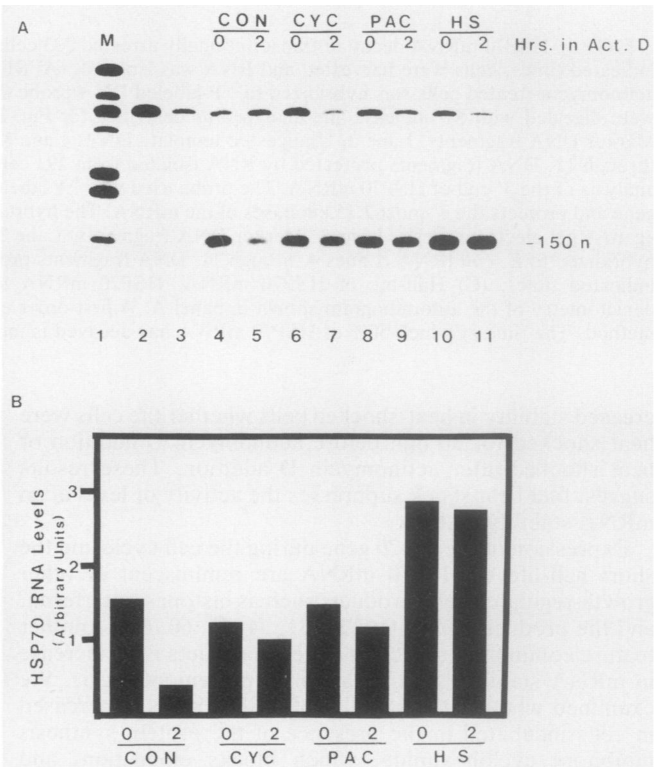

FIG. 8. Effects of protein synthesis inhibitors on HSP70 mRNA stability. Cultures of 293 cells were incubated under the following conditions: no treatment (CON); with 100 μg of cycloheximide per ml for 30 min (CYC); with 0.1 μM pactamycin for 30 min (PAC); at 43°C for 30 min (HS). Each culture was then further incubated the presence of actinomycin for 0 or 2 h; RNA was isolated, and the levels of HSP70 mRNA were determined by S1 nuclease protection and autoradiography. (A) Autoradiogram of the S1 protection assay. Lane 1, Marker DNA fragments. Lane 2, Undigested template DNA. Lane 3, DNA fragments protected after hybridization to *E. coli* tRNA. Lanes 4 through 11, DNA fragments protected by RNA isolated from cells incubated as described. The protected fragment of 150 nucleotides (n) is indicated. (B) Quantification of HSP70 mRNA levels obtained by scanning densitometry of the autoradiogram shown in panel A.

other laboratories which have demonstrated the translation of heat shock mRNAs during the cell cycle and development (9, 10, 49, 59), they are in contrast to studies that have implied a requirement for heat shock for the efficient translation of HSP70 mRNA (20). It is conceivable that growth-regulated expression of HSP70 mRNA may involve altered translational specificity.

We observed heat shock-induced polysome disaggregation concomitant with the release of actin mRNA from polysomes, suggesting that heat shock inhibits translational initiation of at least this cellular mRNA. Our results for HeLa cells are most consistent with the studies of Lindquist (41, 42) and contrast with studies of azetidine-induced stress in HeLa cells (63) and the effects of heat shock on *Drosophila* tissue culture cells (3), which implicate a block at the level of elongation.

We have previously shown that the transcription of the *hsp70* gene is rapidly and transiently activated during heat shock of human and avian cells (4, 71). During heat shock, *hsp70* transcription rates increase rapidly and then decline within 60 min. The high level of HSP70 mRNA in continuously heat-shocked cells is maintained through effects on message stability. A consequence of this form of regulation is that the mechanisms that trigger the heat shock response can be uncoupled from those required to maintain high levels of heat shock protein synthesis. It is also known that heat shock mRNAs rapidly degrade during recovery from heat shock (22, 23, 32). We show here that the instability of HSP70 mRNA is not a feature peculiar to cells recovering from heat shock, but that it is a normal feature of the message in non-heat-shocked cells, both in 293 cells which overproduce HSP70 mRNA and during the HeLa cell cycle (49). Other stresses that induce HSP70 synthesis may also stabilize HSP70 mRNA; for example, treatment of 293 cells with sodium arsenite increased the stability of HSP70 mRNA (data not shown).

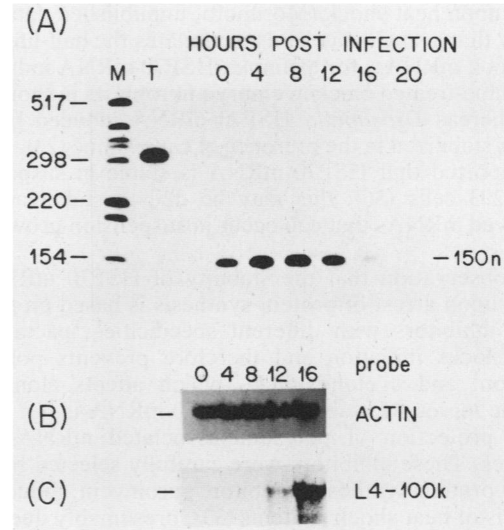

FIG. 10. HSP70, β-actin, and L4 mRNA levels during infection. (A) HSP70 mRNA levels during infection. Cells were infected as described above; at the indicated times postinfection, the cells were harvested and RNA was purified. HSP70 mRNA levels were determined by S1 nuclease protection as described. (B) β-Actin mRNA levels during infection. RNA was isolated from infected cells and analyzed by Northern blot analysis as described for Fig. 1. The blot was probed for the presence of β-actin mRNA as described. (C) Adenovirus L4 RNA levels during infection. The Northern blot shown in Fig. 10B was reprobed for the presence of the adenovirus L4-100K mRNA. The probe used was a plasmid that contains map units 69.5 to 72.8 of the adenovirus genome. This region hybridizes to the L4 transcription unit that encodes the 100K protein.

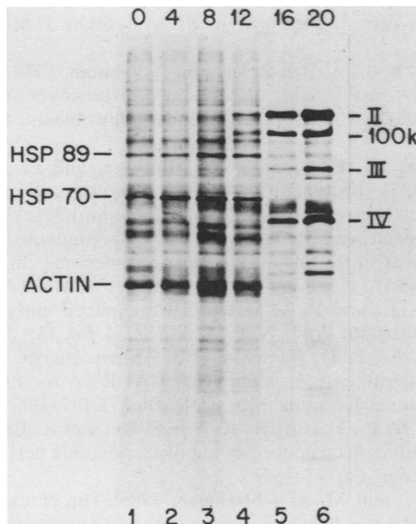

FIG. 9. Protein synthesis patterns in adenovirus-infected 293 cells. Cells were infected at a multiplicity of 10 PFU/cell; at various times postinfection the cells were labeled with [^{35}S]methionine for 15 min. The radiolabeled proteins were analyzed by SDS-PAGE and fluorography. The protein synthesis patterns are given for cells infected for the number of hours indicated at the tops of the lanes (lanes 1 through 6). The positions of the cellular proteins actin, HSP70, and HSP89 are indicated to the left of the fluorogram; the positions of adenovirus proteins II, 100K, III, and IV are indicated to the right.

A role for regulating mRNA stability has been previously proposed to explain the rapid destabilization of HSP70 mRNA which occurs during recovery from heat shock. For *Drosophila melanogaster* it has been suggested that HSP70 mRNA turnover is regulated by the amount of HSP70 that is synthesized during heat shock (23). Our studies reveal that changes in the stability of HSP70 mRNA during heat shock cannot be explained by changes in the level of HSP70 in the cell; the amount of HSP70 in 293 cells is approximately 2 to 4% of the total protein, and its levels do not appreciably

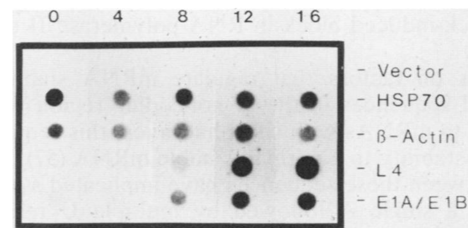

FIG. 11. Transcription in isolated nuclei. Cells were infected as described; at the times indicated at the top (hours postinfection), nuclei were isolated and used in an in vitro run-on transcription assay. The ^{32}P-labeled transcripts were hybridized to cloned DNAs spotted on nitrocellulose. The filters were washed and exposed to X-ray film. The plasmids used for hybridization were: pBR322 (Vector), pH2.3 (HSP70), pHFβA-1 (β-Actin), pSpIVS1 (L4), and pJOLC3 (E1A and E1B).

change upon heat shock (Morimoto, unpublished data). It is unlikely that a single mechanism regulates the half-life of the heat shock mRNAs; for example, HSP70 mRNA induced in canavanine-treated chicken embryo fibroblasts is short-lived (68), whereas *Drosophila* HSP70 mRNA induced by heat shock is stabilized in the presence of canavanine (23). Others have reported that HSP70 mRNA is stable in suspension-grown 293 cells (36); this may be due to stabilization of short-lived mRNAs that can occur in suspension-grown cells (8).

Our observation that the stability of HSP70 mRNA increases upon arrest of protein synthesis is based on the use of two inhibitors with different specificities: pactamycin, which blocks initiation and therefore prevents polysome formation, and cycloheximide, which affects elongation. Thus the increased stability of HSP70 mRNA is not simply due to protection of polysome-associated mRNAs from nucleases. These inhibitors were carefully selected because another protein synthesis inhibitor, puromycin, induces the synthesis of heat shock proteins (33), presumably due to the formation of abnormal proteins resulting from the release of the nascent peptide. The stabilization of HSP70 mRNA by cycloheximide is a feature that is shared with many growth-regulated mRNAs (19, 21, 31, 44, 53, 60, 61).

The effects of adenovirus infection on HSP70 expression are complex. Actin and HSP70 synthesis declines between 12 and 16 h p.i., yet transcription of both genes is not significantly affected. In contrast to the stable maintenance of actin mRNA in a translationally repressed state, HSP70 mRNA is degraded. Since host protein synthesis is blocked during adenovirus infection, we might have expected HSP70 mRNA to exhibit an increased stability. Nevertheless, HSP70 mRNA appeared to be more rapidly degraded in adenovirus-infected cells despite the continued transcription of the gene.

Although our studies do not reveal the mechanism of regulating mRNA stability, it is possible that heat shock affects either the synthesis or the activity of a labile nuclease. New synthesis is probably necessary for the degradation of HSP70 mRNA during recovery from heat shock, as heat-induced HSP70 mRNA is stable in the presence of actinomycin even if the cells are returned to control temperatures (14, 54; see also Fig. 7). The stabilization of HSP70 mRNA by heat shock is not simply due to inhibition of protein synthesis during heat shock. Often protein synthesis in 293 cells is not inhibited by 43°C treatment, yet HSP70 mRNA is stable under these conditions (our unpublished data). Other laboratories have observed that heat shock increases the half-life of histone mRNAs (24). Perhaps increasing mRNA stability is essential to compensate for the heat shock-induced block in RNA polymerase II transcription (25).

Studies on factors that regulate mRNA stability have identified sequences in the 3′ noncoding region which are common to mRNAs with short half-lives; this sequence can confer instability to a normally stable mRNA (57). Comparisons between these sequences have implicated a motif that contains a single A followed by multiple U residues, in particular the sequence AUUUA (57). The 3′ untranslated region of human HSP70 mRNA has one copy of AUUUA as well as several related sequences (35). Although we have no direct evidence that these sequences are necessary for the regulation of human HSP70 mRNA stability, it has been demonstrated that the half-life of a *Drosophila* HSP70 mRNA truncated at the 3′ end (referred to as HSP45) was increased during recovery from heat shock (58). Consequently, HSP70 mRNA might have separate domains at its 5′ and 3′ ends that regulate its translation and turnover. Therefore, it would appear that HSP70 is a good model system for studying factors that influence posttranscriptional regulation of gene expression.

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