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Heat Shock-Induced Translational Control of HSP70 and Globin Synthesis in Chicken Reticulocytes

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Incubation of chicken reticulocytes at elevated temperatures (43 to 45°C) resulted in a rapid change in the pattern of protein synthesis, characterized by the decreased synthesis of normal proteins, e.g., α and β globin, and the preferential and increased synthesis of only one heat shock protein, HSP70. The repression of globin synthesis was not due to modifications of globin mRNA because the level of globin mRNA and its ability to be translated in vitro were unaffected. The HSP70 gene in reticulocytes was transcribed in non-heat-shocked cells, yet HSP70 was not efficiently translated until the cells had been heat shocked. In non-heat-shocked reticulocytes, HSP70 mRNA was a moderately abundant mRNA present at 1 to 2% of the level of globin mRNA. The rapid 20-fold increase in the synthesis of HSP70 after heat shock was not accompanied by a corresponding increase in the rate of transcription of the HSP70 gene or accumulation of HSP70 mRNA. These results suggest that the elevated synthesis of HSP70 is due to the preferential utilization of HSP70 mRNA in the heat-shocked reticulocyte. The heat shock-induced alterations in the reticulocyte protein-synthetic apparatus were not reversible. Upon return to control temperatures (37°C), heat-shocked reticulocytes continued to synthesize HSP70 at elevated levels whereas globin synthesis continued to be repressed. Despite the presence of HSP70 mRNA in non-heat-shocked reticulocytes, we found that continued transcription was necessary for the preferential translation of HSP70 in heat-shocked cells. Preincubation of reticulocytes with the transcription inhibitor actinomycin D or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole blocked the heat shock-induced synthesis of HSP70. Because the level of HSP70 mRNA was only slightly diminished in cells treated with actinomycin D, we suggest two possible mechanisms for the preferential translation of HSP70 mRNA: the translation of only newly transcribed HSP70 mRNA or the requirement of a newly transcribed RNA-containing factor.

Eucaryotic cells respond to elevated temperatures (heat shock) by rapid changes in the pattern of gene expression (for a review, see reference 46). These changes typically occur at two levels, the elevated transcription of a small family of heat shock genes and the preferential translation of heat shock mRNAs. There does not appear to be a single mechanism for the preferential translation of heat shock mRNAs in eucaryotic cells. For example, in yeast cells, heat shock mRNAs are translated whereas the existing non-heat shock mRNAs are degraded (29). In *Xenopus* oocytes the preferential translation of HSP70 is due to preexisting levels of HSP70 mRNA (6). The rapid synthesis of heat shock proteins in *Drosophila* cells is accompanied by the appearance of polysomes containing newly transcribed heat shock mRNAs (32, 33, 49). The heat shock-induced repression of normal cellular protein synthesis is accompanied by a rapid disaggregation of existing polysomes involved in the translation of normal cellular mRNAs (25, 31, 32, 47, 49). The inefficient translation of cellular mRNAs is not due to modification or degradation of the mRNA as shown by the efficient translation of the non-heat shock cellular mRNAs in heterologous cell-free lysates (25, 49).

Although the effects of heat shock on gene expression have been most extensively studied in *D. melanogaster*, the mechanism by which heat shock alters the protein-synthetic apparatus for the preferential utilization of heat shock mRNAs is not understood. Cell-free lysates prepared from heat-shocked *Drosophila* cells translate only heat shock mRNAs, whereas control (non-heat shock) lysates translate both control and heat shock mRNAs (25, 48, 49). The components of the protein-synthetic machinery that appear to be modified may be associated with ribosomes because the addition of crude ribosomes (micrococcal nuclease treated) from non-heat-shocked cells to heat shock lysates allows both control and heat shock mRNAs to be translated (48).

We have previously shown that chicken reticulocytes and lymphocytes respond differently to elevated temperatures in that reticulocytes induce the synthesis of only one heat shock protein, HSP70, whereas all four heat shock proteins are induced in lymphocytes (HSP89, -70, -23, and -22) (R. I. Morimoto and E. J. B. Fodor, J. Cell Biol., in press). We also observed that the existing level of protein synthesis was repressed after exposure to the elevated temperature. In particular, globin synthesis was almost completely repressed after incubation of reticulocytes between 43 and 45°C. This observation provided the opportunity to focus on the mechanism of preferential utilization of a single mRNA, that for HSP70, and the inability of globin mRNA to translate in heat-shocked cells.

We have found that the preferential synthesis of HSP70 and the repression of globin synthesis in heat-shocked reticulocytes are mediated by translational control of protein synthesis. The inability of globin mRNA to translate in heat-shocked cells appears to be due to changes in the protein-synthetic machinery because globin mRNA is neither modified nor degraded. HSP70 mRNA is present in the cytoplasm of non-heat-shocked cells as a moderately abundant mRNA in a translationally repressed state. The level of HSP70 mRNA increases only slightly after heat shock in contrast to the dramatic increase in HSP70 synthesis that occurs in heat-shocked reticulocytes. These results suggest that HSP70

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mRNA is preferentially utilized by the translational apparatus of heat-shocked cells.

MATERIALS AND METHODS

Isolation of reticulocytes, [^{35}S]methionine labeling, and gel electrophoresis. Anemia was induced in white leghorn chickens by a 4-day injection schedule of acetylphenylhydrazine (Eastman Kodak Co.) at 5, 4, 5, and 2 to 4 mg/lb (19). Blood was collected on day 5 into buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.4, 150 mM KCl) containing 1 mg of heparin (168 U/mg; Sigma Chemical Co.) per ml. Cells were washed three times in buffer A, and the buffy coat (leukocytes) was removed by careful aspiration or by separation on Ficoll-Hypaque (Sigma) gradients (9). The cells were resuspended at a concentration of 10^9 cells per ml in buffer A containing 50 mM glucose. The level of reticulocyte enrichment was determined by light microscopy after staining with Wright's Giemsa stain. Typically, reticulocytes were 70 to 90% of the erythrocyte population.

Reticulocytes (5×10^7 cells) were suspended at a concentration of 10^9 cells per ml in 1.5-ml Eppendorf tubes and incubated in buffer A containing 50 mM glucose at the various temperatures and times indicated. Protein synthesis was measured by incubating reticulocytes for 30 min at 37°C in methionine-deficient medium containing 50 μCi of [^{35}S]methionine (Amersham Corp.) per ml. The radioactive medium was removed, and the cells were washed with buffer A and immediately frozen at −80°C. Changes in protein synthesis were measured by analysis on 10 or 12.5% polyacrylamide gels (26). The rates of protein synthesis were measured by trichloroacetic acid precipitation of duplicate 5-μl samples of cell lysates. Samples were treated at 90°C for 5 to 10 min and chilled on ice, and the protein precipitate was collected on GFC filters. The synthesis of HSP70 and globin was measured directly by excising the respective bands from fluorographed gels (27) and measuring the radioactivity by liquid scintillation counting.

RNA isolation and characterization. RNA was isolated by a modified guanidine thiocyanate procedure (12) followed by hot phenol extraction (17). Chicken reticulocytes were incubated at control or heat shock temperatures, pelleted by centrifugation, and homogenized in at least 5 volumes of 4 M guanidine thiocyanate containing 50 mM Tris-hydrochloride (pH 7.6), 10 mM EDTA, 1% (vol/vol) 2-mercaptoethanol, and 2% (wt/vol) Sarkosyl. Equal volumes of hot (60°C) phenol, 0.1 M sodium acetate (pH 5.5), and chloroform-isoamyl alcohol (24:1) were added. The RNA was extracted at 60°C for 10 min, chilled on ice, and centrifuged to separate the phases. The aqueous phase was recovered, reextracted twice more with phenol and chloroform at 60°C and once with chloroform at room temperature, and precipitated with 2.5 volumes of ethanol. Total polyadenylated [poly(A)$^+$] RNA was purified by using oligodeoxythymidylate-cellulose chromatography (2).

Up to 1 μg of poly(A)$^+$ RNA was translated in 25 μl of micrococcal nuclease-treated rabbit reticulocyte lysates (40) or in 100 μl of wheat germ extracts (43). Comparisons of in vitro translation products from mRNA isolated from control and heat-shocked cells were done with suboptimal concentrations of poly(A)$^+$ mRNA as determined by titration in the mRNA-dependent translation systems. The final concentrations of components in the rabbit reticulocyte translation assay were as follows: 40% (vol/vol) reticulocyte lysate; 120 μg of creatine phosphokinase (type I; Sigma) per ml; 24 μM hemin; 20 mM HEPES-KOH, pH 7.4; 0.5 mM spermidine trihydrochloride; 8 mM creatine phosphate; 2 mM dithiothreitol; 0.025 mM concentrations of each amino acid except methionine (neutralized with HCl); 1 mCi of [^{35}S]methionine per ml; 60 mM KCl; and 0.08 mM $Mg(OAc)_2$. The reticulocyte translation assays were incubated at 30°C for 90 min. The final concentrations of components in the wheat germ translation assay were as follows: 40% (vol/vol) wheat germ extract; 1 mM ATP; 0.2 mM GTP; 6 mM creatine phosphate; 80 μg of creatine phosphokinase per ml; 0.1 mM spermine tetrahydrochloride; 2 mM dithiothreitol; 0.1 mM concentrations of each amino acid except methionine; 0.1 mCi of [^{35}S]methionine per ml; 20 mM HEPES-KOH, pH 7.4; 110 mM KOAc; and 1 mM $Mg(OAc)_2$. The wheat germ translation assays were incubated at 28°C for 60 min.

RNA was analyzed by Northern blot analysis (30, 51) after denaturation by glyoxal and dimethyl sulfoxide (34). Glyoxalated RNA was electrophoresed on a 1.4% agarose gel in 10 mM sodium phosphate buffer, pH 7.0, and then transferred to nitrocellulose. The filter was baked, washed in a solution containing 6× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt solution, and 50 μg of tRNA per ml, and then hybridized at 42°C to ^{32}P-labeled (nick-translated) plasmids containing either the chicken β-globin gene pβ1BR15 (16) or the chicken HSP70 gene pC1.8 (Morimoto et al., in press). Plasmid pC1.8 is a 1.8-kilobase (kb) *Hin*dIII subclone of a genomic recombinant (C411) isolated by screening a genomic chicken library with the *Drosophila* HSP70 gene. This subclone contains 0.7 kb of upstream sequences and 1.1 kb of the chicken HSP70 gene from the ATG initiator codon and extending to the end of the insert at amino acid 326. The HSP70 gene sequence in pC1.8 is single copy per haploid chicken genome. After hybridization, blots were washed for 30 to 60 min at 65°C in 6× SSC–0.1% SDS followed by a wash in 1× SSC–0.1% SDS, air-dried, and exposed to X-ray film.

Isolation of reticulocyte nuclei, in vitro transcription, and purification of [^{32}P]UTP-labeled transcripts. Samples of reticulocytes or MSB cells in buffer A were placed at 37°C (control temperature) or at the heat shock temperatures (41 to 47°C). After the desired incubation, cells were removed and nuclei were isolated (22). Cells were lysed in reticulocyte standard buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$) containing 0.5% Nonidet P-40 (Sigma). Cell lysis was monitored by light microscopy and from the whitish color of the nuclear pellet. Nuclei were frozen at −80°C in 40% glycerol–50 mM Tris (pH 8.3)–5 mM $MgCl_2$–0.1 mM EDTA at a concentration of 2×10^9 nuclei per ml, which is equivalent to 2 mg of genomic DNA per ml (assuming 10^{-12} g of DNA per haploid chicken genome).

In vitro runoff transcription in isolated nuclei was performed by using modifications of published procedures (22). Nascent RNA transcripts were allowed to elongate in reaction mixtures containing 25 mM HEPES (pH 7.5), 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 5% glycerol, 350 μM ATP, 350 μM GTP, 350 μM CTP, 0.4 μM UTP, [^{32}P]UTP, and 10^9 nuclei per ml with incubation at 26°C for 45 min. Typically, reactions contained 200 μg (100 μl of nuclei) of DNA and 200 μCi (2,000 Ci/mmol; ICN) of [^{32}P]UTP for RNA used for hybridizations. Reactions were stopped by the addition of RNase-free DNase and further incubation for 15 min at 37°C, after which the reactions were diluted in buffer B containing 2% SDS, 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 8. The cell lysate was passed through a sterile 27-gauge syringe and extracted twice with equal volumes of phenol and chloroform and once with chloroform alone. The

final aqueous phase was precipitated at −20°C with 0.25 M ammonium acetate and 2.5 volumes of ethanol.

Labeling with [^{3}H]uridine. In vivo labeling of reticulocytes with [^{3}H]uridine was carried out in 10 mM HEPES (pH 7.4)–150 mM KCl–50 mM glucose supplemented with 50 μCi of [^{3}H]uridine (42 Ci/mmol; ICN) per ml at either control (37°C) or heat shock (43°C) temperature (18). At each time, duplicate samples were removed and the total radioactivity incorporated was measured by trichloroacetic acid precipitation in the presence of 100 μg of *Escherichia coli* tRNA per ml. In addition, RNA was isolated from labeled cells after incubation at 37 or 43°C for 120 min and incubation with [^{3}H]uridine for 60 min at 37°C.

RNA-DNA dot hybridization. Various recombinant DNA clones containing the actin (pA1; 13), β-globin (pβ1BR15; 16), and the HSP70 genes (pC1.8; Morimoto et al., in press) were spotted, along with the plasmid vector pAT153 as a negative control, onto nitrocellulose (24). The plasmid DNAs were linearized by sonication to an average size of 500 base pairs, denatured by 0.4 N NaOH, and neutralized by the addition of an equal volume of 2 M ammonium acetate. Dot blot analysis of total poly(A)$^+$ RNA isolated from control or heat-shocked cells were performed by denaturation of the mRNA with formaldehyde (30) as preparation for binding to nitrocellulose. The nitrocellulose filter was soaked in water and placed on a Minifold (Schleicher & Schuell), and plasmid DNAs were spotted and washed with 1 M ammonium acetate. The nitrocellulose was washed in 4× SSC, air-dried, and shaken in 2× Denhardt solution for 1 h, air-dried again, and baked under vacuum at 80°C for 2 h. Blots were prehybridized in 50% formamide–6× SSC–5× Denhardt solution–0.2% SDS–100 μg of carrier tRNA per ml for 16 h at 42°C. The hybridization mixture contained 1 part 50% (wt/vol) dextran sulfate and 4 parts hybridization buffer containing the radiolabeled probe. The ^{32}P-labeled in vitro transcribed RNA was denatured at 65°C for 5 min, cooled on ice, and added to the hybridization buffer just before addition to the blot. For the RNA dot blot, ^{32}P-labeled DNA hybridization probes (pβ1BR15 and pC1.8) were prepared as previously described. Hybridization was conducted for 72 h. Blots were washed in three changes of 2× SSC–0.1% SDS at 65°C. Blots were exposed to XAR-5 Kodak film at −70°C with a Dupont Cronex Lightning-plus intensifying screen. Direct quantitation of hybridized material was obtained by excising the nitrocellulose dots and counting by liquid scintillation.

RESULTS

Induction of HSP70 synthesis and repression of globin synthesis in heat-shocked chicken reticulocytes. Chicken reticulocytes respond to elevated temperatures by the induced synthesis of a single heat shock protein, HSP70, and the repression of globin synthesis. Incubation of reticulocytes at elevated temperatures (41 to 45°C) has three effects on protein synthesis (Fig. 1): (i) repression of overall levels of protein synthesis, (ii) repression of α- and β-globin synthesis

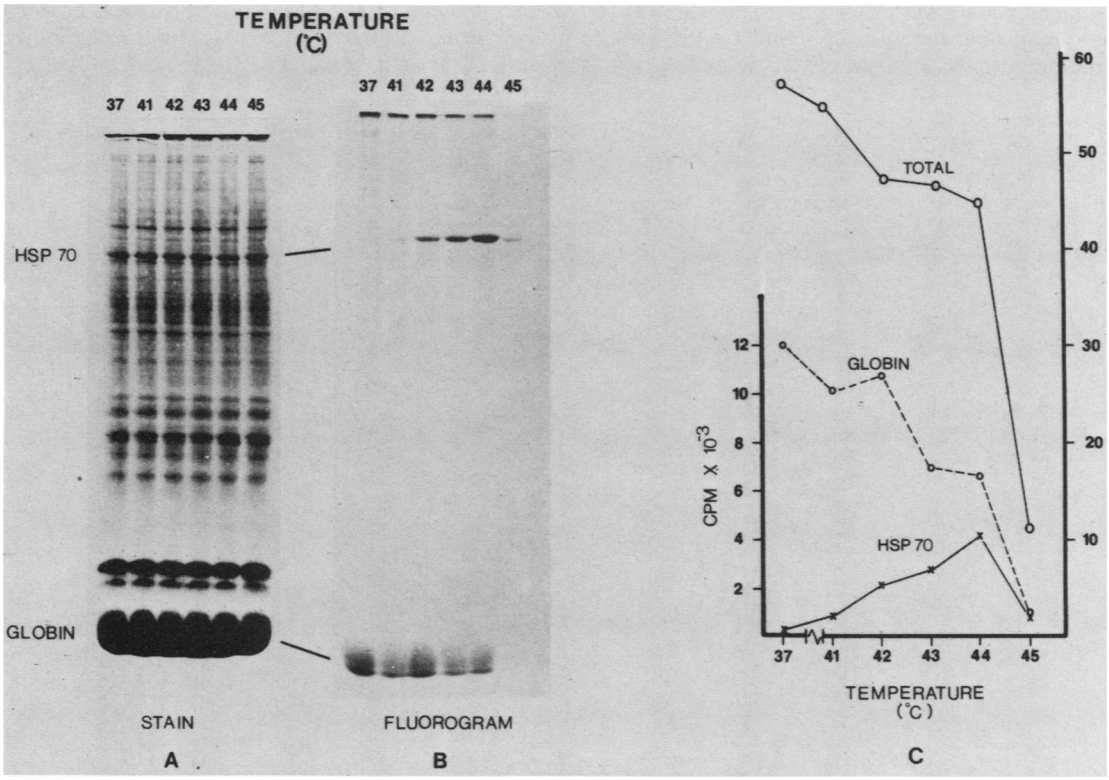

FIG. 1. Effect of incubation at elevated temperatures on protein synthesis in reticulocytes. Reticulocytes (5 × 10^7 cells) isolated from Ficoll-Hypaque gradients were incubated at 37, 41, 42, 43, 44, or 45°C for 30 min, pelleted, suspended in 200 μl of methionine minus Dulbecco modified Eagle medium containing 50 μCi of [^{35}S]methionine per ml, and incubated at 37°C for 30 min. The cells were collected, lysed in SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis or assayed for total protein synthesis by trichloroacetic acid precipitation of duplicate samples. (A) Coomassie brilliant blue-stained gel. Proteins corresponding to HSP70 and globin are marked. (B) Fluorogram of gel in (A). (C) Quantitation of total protein synthesis (○——○). The right ordinate indicates levels of total protein synthesis. The levels of globin (○– – –○) or HSP70 (×——×) synthesis were determined by excising the corresponding bands from fluorograms and measuring the radioactivity by liquid scintillation counting.

after incubation at 43 to 45°C, and (iii) selective induction in the synthesis of one heat shock protein, HSP70. Because each lane on the SDS-polyacrylamide gel shown in Fig. 1 corresponds to equivalent amounts of reticulocyte cell lysates, we can compare the relative rates of synthesis of globin and HSP70. This type of comparison makes the assumption that the pool levels of methionine and the rate of uptake of [^{35}S]methionine do not change appreciably in heat-shocked reticulocytes.

The transient incubation of reticulocytes at elevated temperatures does not alter the Coomassie blue-stained protein patterns (Fig. 1A). HSP70 is one of the major nonglobin proteins of the normal chicken reticulocyte (Morimoto and Fodor, in press). The synthesis of HSP70 is typically induced 20-fold after a 30-min incubation at 43 to 44°C, whereas globin synthesis is decreased by 50% (Fig. 1B and C). The repressive effects of heat shock on protein synthesis are also seen for other proteins synthesized in reticulocytes, e.g., two proteins of 200,000 daltons, presumably spectrin. The overall level of protein synthesis at 42 to 44°C is repressed 20%. After incubation at 45°C, total protein synthesis is repressed 84%. Under these conditions only HSP70 synthesis continues, albeit at reduced levels.

The kinetics of HSP70 induction and the repression of globin synthesis at 43°C are shown in Fig. 2. Reticulocytes were incubated at 43°C for up to 3 h, and the subsequent changes in the rates of total protein synthesis and the specific effects on HSP70 and globin synthesis were examined (Fig. 2B). The synthesis of HSP70 is transiently induced, reaching maximal levels after a heat shock of 30 to 60 min. Globin synthesis is initially repressed by the shift to 43°C and continues to be synthesized at decreased levels such that after 3 h at 43°C only 20% of the control levels of globin are synthesized.

Quantitative analysis of globin mRNA from normal and heat-shocked reticulocytes. There are three likely explanations for the altered pattern of in vivo protein synthesis, specifically, of the decreased translation of globin mRNA in heat-shocked cells. Incubation at elevated temperatures could affect the stability of globin mRNA, thus decreasing the cytoplasmic levels of this mRNA. This can be determined by Northern blot analysis. The second possibility, that globin mRNA is not degraded but becomes structurally modified and no longer translatable, can be examined by in vitro translation. The third possibility is that globin mRNA is neither modified nor degraded but is incapable of translating in vivo due to modifications of the protein-synthetic machinery.

Reticulocytes were either maintained at control temperatures or heat shocked for increasing times (30, 60, 120 min) at 43°C. Total poly(A)$^+$-containing mRNA was isolated by chromatography on oligodeoxythymidylate-cellulose and analyzed by in vitro translation in rabbit reticulocyte lysates or wheat germ extracts to examine translational activity. The cytoplasmic poly(A)$^+$ mRNA from control and heat-shocked cells was titrated in the mRNA-dependent rabbit reticulocyte system to establish translation conditions, using suboptimal levels of mRNA. Equivalent amounts of globin are synthesized in reticulocyte lysates or wheat germ extracts in the presence of poly(A)$^+$ mRNA isolated from control or heat-shocked reticulocytes (Fig. 3). Because similar results are obtained with both in vitro translation systems, the likely conclusion is that the levels and translatability of globin mRNA are not reduced by heat shock.

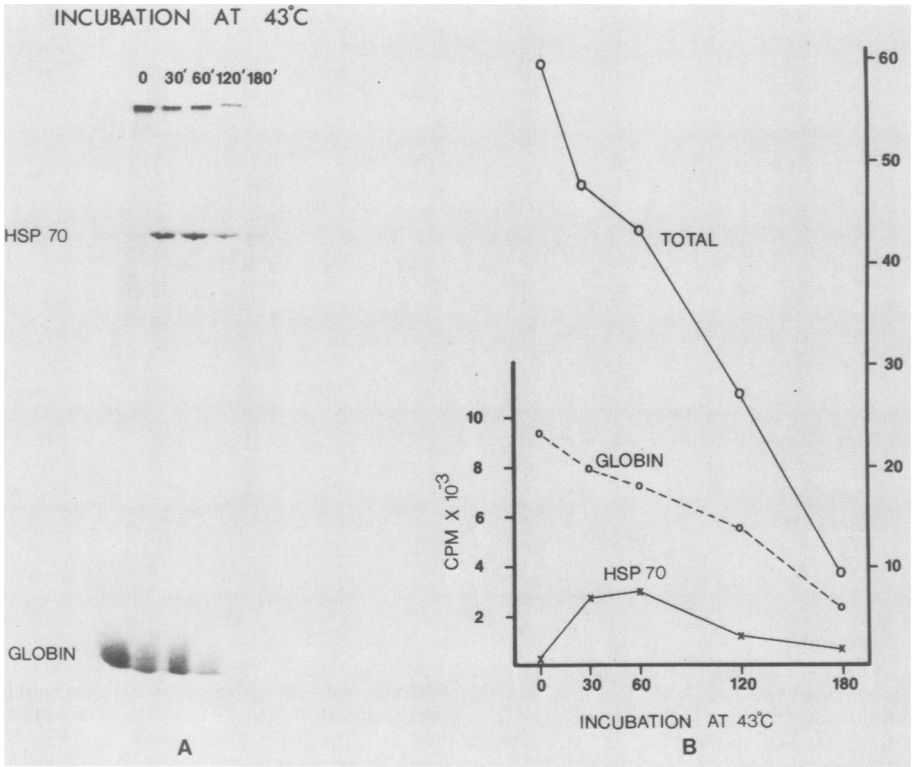

FIG. 2. Effect on protein synthesis of incubation of reticulocytes at 43°C for increasing times. Reticulocytes were incubated for 30, 60, 120, or 180 min at 43°C or for 30 min at 37°C and pelleted, and protein synthesis was measured as described for Fig. 1. (A) Fluorogram of SDS-polyacrylamide gel; (B) quantitation of total protein synthesis (—○—), globin (– –○– –), and HSP70 synthesis (×——×).

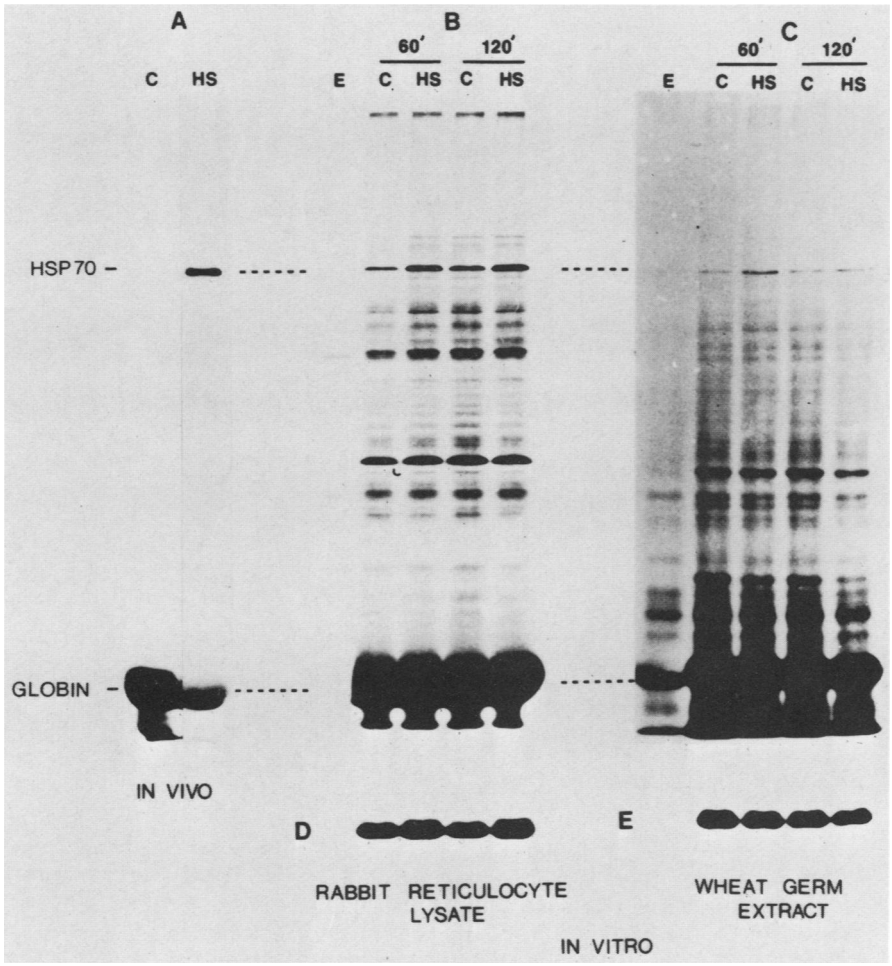

FIG. 3. In vitro translation of mRNA isolated from control (C) or heat shock (HS) reticulocytes in rabbit reticulocyte lysates and wheat germ extracts. Total poly(A)$^+$ mRNA was isolated from control (37°C) or heat-shocked (43°C for 60 or 120 min) reticulocytes as described in the text and translated in (B) mRNA-dependent rabbit reticulocyte lysate or (C) wheat germ extracts; the products were analyzed on a 12.5% polyacrylamide gel containing SDS and fluorographed. Lane E in (B) and (C) corresponds to endogenous protein synthesis in reticulocyte and wheat germ systems, respectively. A smaller sample (10%) of the in vitro translation extracts shown in (B) and (C) was electrophoretically analyzed and the regions corresponding to the globin proteins are shown in (D) and (E). The amount of poly(A)$^+$ mRNA used for the 60- the 120-min samples in the reticulocyte lysate was inadvertently lower than the adjacent controls. For comparison, in (A) the in vivo pattern of protein synthesis of control and heat-shocked (45°C) reticulocytes is shown.

The yield of mRNA extracted from control and heat-shocked cells was compared by Northern blot analysis and the results reveal no detectable loss of globin mRNA in the heat-shocked cells (Fig. 4). This is evident from a quantitative analysis of the amount of globin mRNA isolated from equal numbers of control or heat-shocked reticulocytes. Reticulocyte mRNA was glyoxalated and electrophoretically separated on a neutral agarose gel, transferred to nitrocellulose filters, and allowed to hybridize to ^{32}P-labeled pβ1BR15 (adult chicken β-globin gene; 16). Approximately equal levels of globin mRNA (0.75 kb) are present in the poly(A)$^+$ mRNA isolated from control or heat-shocked cells (Fig. 4A).

We conclude from these experiments that the exposure of reticulocytes to elevated temperatures does not affect the level or the ability of globin mRNA to be in vitro translated. Therefore, the decreased synthesis of globin is likely to be due to the inability of globin mRNA to be efficiently translated on the altered protein-synthetic apparatus.

Level of HSP70 mRNA in control and heat-shocked reticulocytes. An estimate of the relative abundance of HSP70 mRNA in control and heat-shocked reticulocytes can be obtained by in vitro translation of poly(A)$^+$ mRNA and by Northern blot analysis. The amount of HSP70 synthesized in vitro by poly(A)$^+$ mRNA isolated from heat-shocked cells increases only 2-fold over the levels found in non-heat-shocked cells (Fig. 3) and is small in comparison to the 20-fold increase in the rate of HSP70 synthesis (Fig. 2). In view of the dramatic increase in HSP70 synthesis after heat shock, we examined the levels of HSP70 mRNA in total poly(A)$^+$ mRNA isolated from control and heat shock reticulocytes by hybridization of Northern blots with the cloned chicken HSP70 gene. The results shown in Fig. 4A indicate that HSP70 mRNA (2.6 kb) is present in control cells and that the level of HSP70 mRNA increases approximately twofold after heat shock. For comparison, the amount of HSP70 mRNA in heat-shocked transformed chicken lymphocytes (Marek's disease virus-transformed lymphocytes [1]) increases approximately 20- to 30-fold (Fig. 4A). It follows from these results that the dramatic increase in HSP70 synthesis observed after heat shock is not due to a

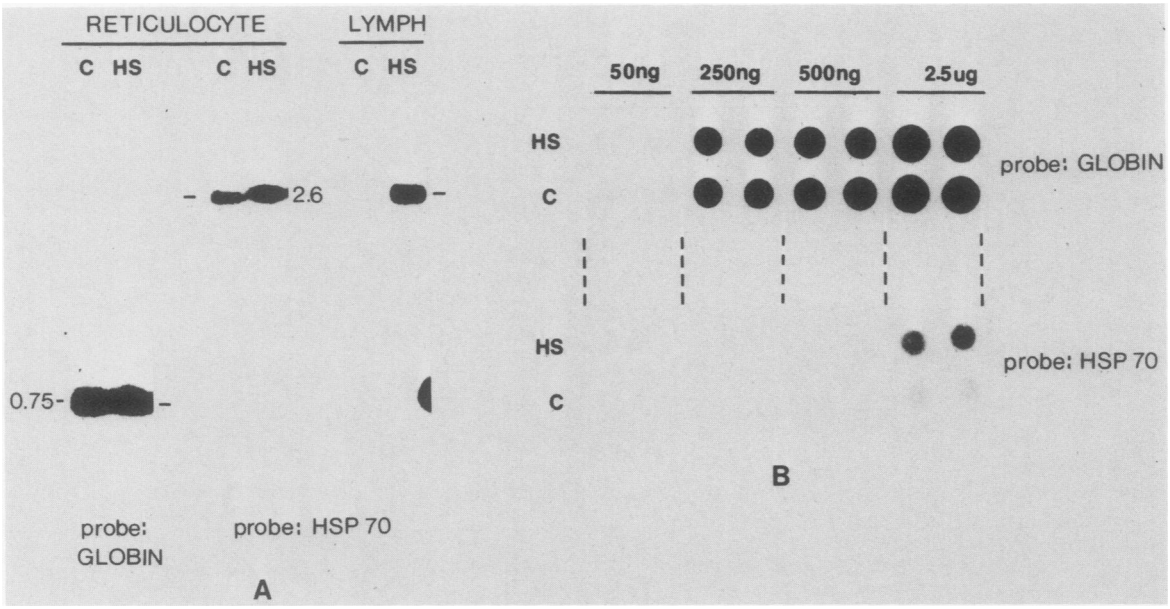

FIG. 4. Hybridization analysis of total poly(A)$^+$ RNA isolated from control (C) and heat-shocked (HS) reticulocytes with the cloned chicken β-globin or HSP70 gene. (A) Total poly(A)$^+$ RNA from control (37°C) and heat-shocked (45°C for 60 min) reticulocytes was glyoxalated and electrophoretically separated on a neutral agarose gel as described in the text; the RNA was transferred to nitrocellulose and hybridized to ^{32}P-labeled pβ1BR15, 3-h exposure (β-globin), and ^{32}P-labeled pC1.8, 28-h exposure (HSP70 gene). The size of each transcript (HSP70, 2.6 kb; globin, 0.75 kb) is shown. The poly(A)$^+$ RNA from control and heat-shocked (43°C for 60 min) MSB lymphocytes was analyzed as described in the text and hybridized to ^{32}P-labeled pC1.8, 5-h exposure. (B) RNA dot hybridization of total cytoplasmic RNA from control and heat-shocked cells to ^{32}P-labeled pβ1BR15 and pC1.8. The amounts of mRNA loaded onto each set of duplicate dots are shown. The specific activities of the ^{32}P-labeled DNA hybridization probes were similar (4×10^7 to 5×10^7 cpm/μg of DNA, 2-h exposure).

rapid accumulation of HSP70 mRNA but rather to a greatly increased mRNA utilization. The results of Northern blot analysis and in vitro translation both demonstrate that HSP70 mRNA is present in normal reticulocytes and that the levels do not change significantly after heat shock.

We compared the relative levels of HSP70 and β-globin mRNA in cytoplasmic mRNA isolated from control and heat-shocked reticulocytes by hybridization of the respective cloned gene sequences to RNA dot blots. The results show that HSP70 mRNA is present at 1 to 2% of the level of β-globin mRNA (Fig. 4B) and that the level of HSP70 mRNA increases slightly after heat shock. We conclude from this result that HSP70 mRNA is a moderately abundant mRNA in the cytoplasm of non-heat-shocked reticulocytes.

Transcription of the HSP70 gene is not induced by heat shock. The results of RNA-DNA hybridization (Fig. 4) show that the amount of HSP70 mRNA in heat-shocked cells does not increase proportionally to the increase in HSP70 synthesis. Because Northern blot analysis measures only the total amount of mRNA and does not distinguish between preexisting and newly synthesized mRNA, we examined the rates of transcription on the HSP70 gene in control and heat-shocked reticulocytes. The transcriptional activity of a specific gene can be measured by using in vitro transcribed RNA from isolated nuclei to probe cloned gene sequences. The elongation of preinitiated nascent transcripts allows us to compare directly the rates of transcription of the HSP70 gene in control and heat-shocked cells.

We established the parameters of the isolated nuclei in vitro transcription system by demonstrating that the overall levels of [^{32}P]UTP incorporation into reticulocyte nuclear RNA increased with the time of incubation at 26°C. RNA synthesis is inhibited 75% by the addition of 2 μg of α-amanitin per ml and almost completely after addition of 10 μg of actinomycin D per ml (Fig. 5A). These results confirm that the predominant transcriptional activity in isolated nuclei from chicken reticulocytes is RNA polymerase II-dependent transcription (19, 22).

The effects of heat shock on overall transcription were examined by incubating reticulocytes at elevated temperatures (37 to 47°C) for 60 min, isolating nuclei, and allowing preinitiated transcripts to elongate in the reaction mixture containing [^{32}P]UTP. The overall level of transcription was decreased 80% by heat shock (Fig. 5B). Furthermore, continued incubation at 43 or 45°C on transcription revealed that α-amanitin-sensitive transcription is repressed by >90% upon incubation at either temperature (Fig. 5C). We conclude from these results that α-amanitin-sensitive RNA polymerase II transcription is repressed rapidly by heat shock, and furthermore, the levels of overall transcription decrease either with continued incubation at a given elevated temperature or with increasing elevated temperatures. These results are similar to the effects of heat shock on RNA polymerase II-dependent transcription observed in *Drosophila* cells (35).

The specific effects of heat shock on the transcription of the chicken actin, globin, and HSP70 genes were examined by hybridization of ^{32}P-labeled in vitro transcribed RNA to each cloned DNA. After the filters were exposed to X-ray film, the amounts of radiolabeled transcripts that hybridized to each DNA were measured directly by liquid scintillation counting. The HSP70 gene is transcribed in nuclei from control cells (Fig. 6). After heat shock, transcription of HSP70 increased slightly to a level 1.5-fold greater than non-heat shock levels. The level of transcription of the two control genes, actin (pA1 [13]) and β-globin (pβ1BR15 [16]), decreased after heat shock (30% for actin, 50% for globin). The absence of significant induction of the HSP70 gene in

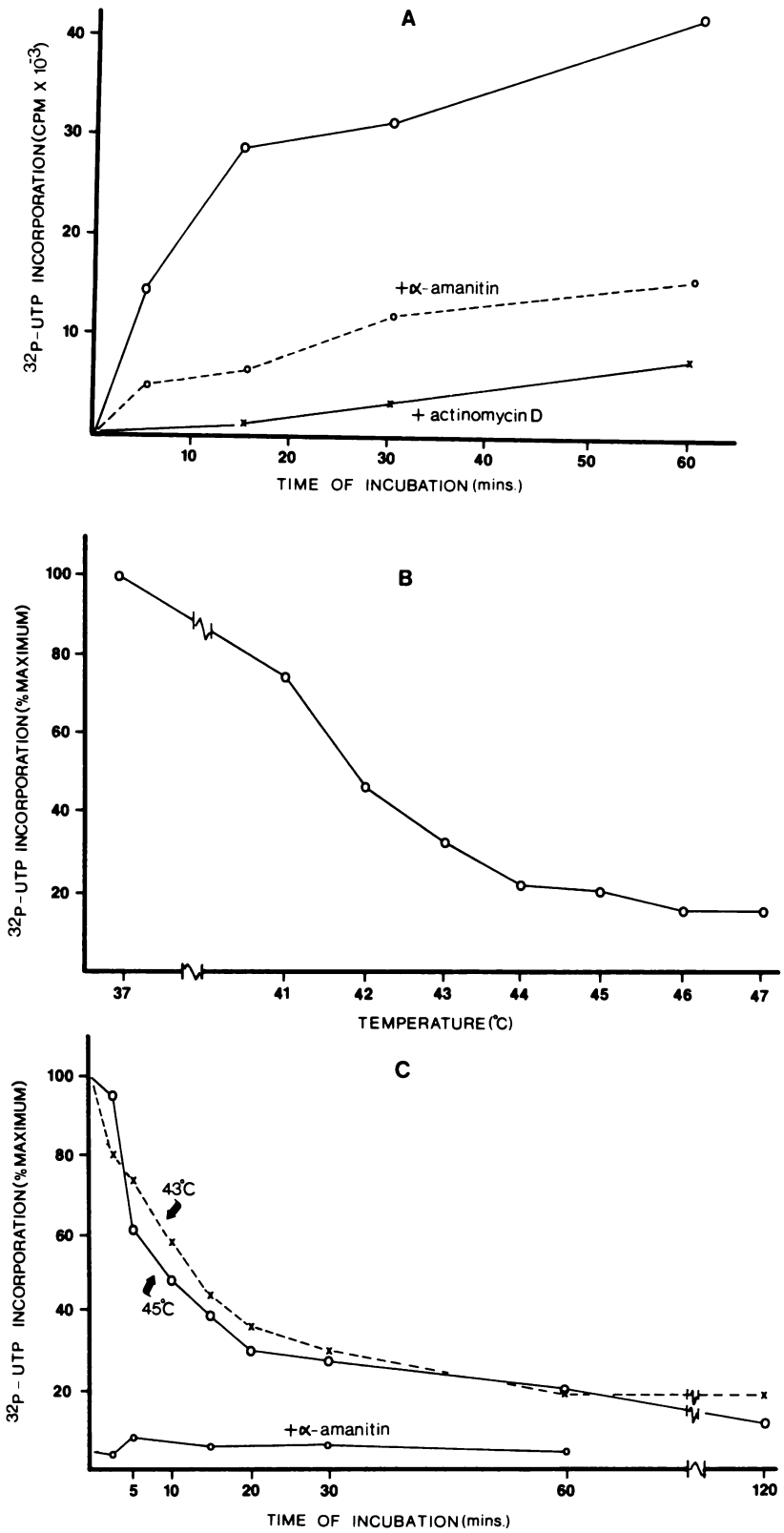

FIG. 5. Transcription in isolated reticulocyte nuclei; effects of heat shock on transcription. (A) Nuclei were prepared from control reticulocytes and transcription was continued in the presence of [^{32}P]UTP as described in the text. At the indicated incubation times, duplicate samples were removed and spotted onto Whatman DE81 filters; unincorporated label was removed by washing with 0.5 M Na_2HPO_4. The filters were dried and radioactivity was measured by scintillation counting. Reactions were also performed in the presence of 2 μg of α-amanitin or 10 μg of actinomycin D per ml. (B) Reticulocytes were incubated at the indicated temperatures for 60 min and nuclei were isolated. Nuclear runoff transcriptions were carried out, duplicate samples were taken, and incorporation into RNA was measured as described in the text. The data shown are the average of two experiments. (C) Reticulocytes were incubated at 43 or 45°C for up to 120 min. Nuclei were isolated at the indicated times and runoff transcription in isolated nuclei was measured. Parallel experiments with 2 μg of α-amanitin per ml were included. The data shown are the average of two experiments.

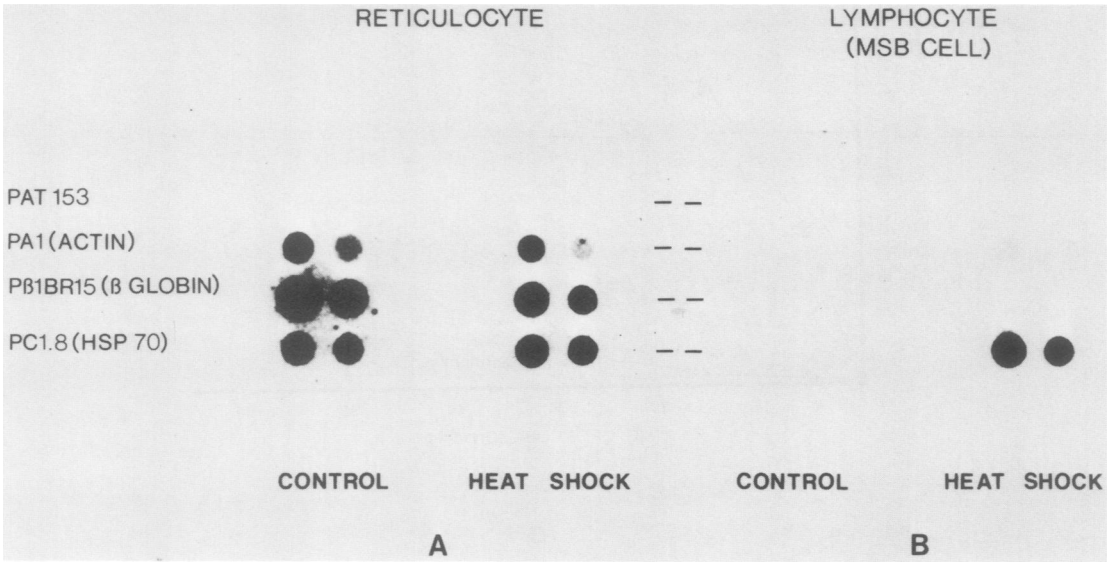

FIG. 6. Hybridization of [^{32}P]UTP-labeled in vitro transcribed RNA from reticulocyte or lymphocyte (MSB cells) nuclei to cloned genes. RNA was isolated from nuclei of control or heat-shocked (43°C for 60 min) cells as described in the text and allowed to hybridize for 48 h to sheared and denatured plasmids containing the plasmid vector pAT153, actin (pA1), β-globin (pβ1BR15), and HSP70 (pC1.8). The two DNA dots correspond to 1 μg and 250 μg of each plasmid DNA.

reticulocytes is consistent with our previous results of Northern hybridizations and isolated nuclei in vitro transcriptions. The 2-fold induction of HSP70 mRNA in heat-shocked reticulocytes contrasts with the 20-fold induction of HSP70 transcription observed in transformed chicken lymphocytes (Fig. 6). Furthermore, the transcription of the globin gene is unique to reticulocyte nuclei, as expected.

We also demonstrated that the HSP70 gene is transcribed in intact control and heat-shocked cells by hybridization of newly synthesized [^{3}H]uridine-labeled RNA to the cloned genes. The overall effects of heat shock on the level of [^{3}H]uridine incorporation in normal and heat-shocked reticulocytes was initially examined. Reticulocytes were incubated at 37 or 43°C for 2 h and total transcription was measured by incorporation of [^{3}H]uridine. RNA synthesis in heat-shocked cells is repressed to approximately 30% of control levels (Fig. 7). The [^{3}H]uridine-labeled RNA isolated from control and heat-shocked cells was hybridized to filters containing the actin, globin, and HSP70 genes. Similar levels of ^{3}H-labeled RNA transcribed in control or heat-shocked reticulocytes hybridized to the HSP70, actin, or globin genes (Table 1). These results demonstrate that the HSP70 gene is

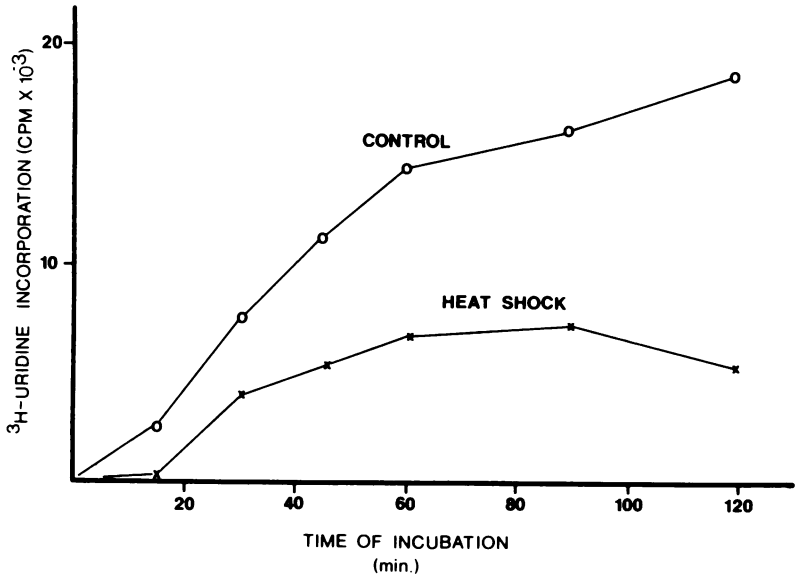

FIG. 7. Effect of heat shock (43°C) on RNA synthesis in reticulocytes as measured by [^{3}H]uridine incorporation. Identical cultures of reticulocytes were incubated at 37 or 43°C. At the indicated times duplicate samples were removed and total incorporated radioactivity was measured by trichloroacetic acid precipitation in the presence of 100 μg of *E. coli* tRNA per ml as carrier.

TABLE 1. Hybridization of [^{3}H]uridine-labeled RNA isolated from control and heat-shocked reticulocytes to chicken actin, globin, and HSP70 genes[a]

DNA bound to nitrocellulose filter	cpm	
	Control	Heat shock
PAT153 (plasmid vector)	2	5
PA1 (actin)	11	10
PB1BR15 (globin)	90	85
PC1.8 (HSP70)	127	146

[a] Reticulocytes (10^9 cells) were suspended in medium containing 50 μCi of [^{3}H]uridine per ml and incubated at control (37°C) or heat shock (43°C) temperature; total RNA was isolated as described in the test and used for hybridization to the various plasmids containing vector or gene sequences. The input counts were 7,880 cpm for control and 3,460 cpm for heat shock. Similar ratios of hybridization to globin and HSP70 gene sequences were observed with nitrocellulose filters containing fourfold less bound DNA.

transcribed in reticulocytes maintained at control and heat shock temperatures and that the level of transcription is induced slightly after heat shock.

The results of all three experiments lead us to conclude that the HSP70 gene is transcribed and that steady-state levels of HSP70 mRNA are maintained in the normal reticulocyte. The rate of transcription of the HSP70 gene does not change significantly after heat shock.

Heat shock-induced translation of HSP70 is blocked by transcriptional inhibitors. We expected that the increased synthesis of HSP70 is due to the preferential utilization of HSP70 mRNA by a heat shock-induced modification of the translational machinery. Because HSP70 mRNA exists in non-heat-shocked cells, we predicted that the preferential translation of HSP70 does not require continued transcription. We tested this prediction by incubating reticulocytes with the transcriptional inhibitor actinomycin D (40a) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (49a) before heat shock and examined for HSP70 synthesis. The increased synthesis of HSP70 that typically occurs in response to heat shock is blocked by incubation in 10 μg of actinomycin D per ml or 70 μM DRB (Fig. 8A). These results suggest that the synthesis of HSP70 in heat-shocked reticulocytes requires continued transcription and the repression of globin synthesis is not sufficient for the induced synthesis of HSP70. These conclusions are limited by other possible effects of these transcription inhibitors.

One possible explanation for the requirement for continued transcription for HSP70 synthesis is that the existing levels of HSP70 mRNA are degraded. Therefore, in cells treated with actinomycin D, the lack of HSP70 synthesis could be explained if HSP70 mRNA were no longer present in the cytoplasm. This was tested by isolating cytoplasmic poly(A)$^+$ RNA from cells maintained at 37°C or heat shocked (43°C) in the presence or absence of 10 μg of actinomycin D per ml and determining the level of globin and HSP70 mRNA by in vitro translation in reticulocyte lysates and by Northern blot analysis. The amount of HSP70 mRNA in cells treated with actinomycin D does not differ significantly from control levels and is decreased slightly, as expected, from levels found in heat-shocked cells (Fig. 8B and C). Therefore, we can conclude that the block in HSP70 synthesis is not due to the absence of HSP70 mRNA.

DISCUSSION

Chicken reticulocytes respond to heat shock by the preferential translation of only one heat shock protein, HSP70, and the repression of globin synthesis. These changes in protein synthesis reflect a heat-induced translation control of protein synthesis that requires the efficient utilization of HSP70 mRNA. In contrast to *Drosophila* cells in which the preferential synthesis of heat shock proteins is preceded by the elevated transcription of heat shock genes, we find in chicken reticulocytes that the HSP70 gene is transcribed in normal cells and that the level of transcription is not induced significantly by heat shock. The HSP70 mRNA in non-heat-shocked cells is maintained in a translationally repressed state in the cytoplasm. Chicken reticulocytes share similarities with *Xenopus* oocytes in that the preferential translation of HSP70 after heat shock is most easily explained by the presence of HSP70 mRNA in the cytoplasm of non-heat-shocked cells (6).

Much of our understanding of heat-induced alterations in protein synthesis has come from laboratories studying *Drosophila* spp. in which it has been possible to reproduce some of the in vivo observations of translational control in crude lysates (25, 48, 49). These studies have demonstrated that lysates prepared from heat-shocked *Drosophila* cells preferentially translate heat shock mRNAs. The translation of control mRNAs in heat shock lysates requires the addition of crude ribosomes from non-heat-shocked cells. These results suggest that the putative discriminatory factor is likely to be associated with ribosomes. Attempts to identify changes in ribosomes associated with heat shock have focused on changes in the phosphorylation of the ribosomal protein S6 (20, 39, 45). However, it is unlikely that modifications of S6 alone are sufficient to explain both the preferential translation of heat shock proteins and the subsequent recovery of normal protein synthesis because dephosphorylation of S6 was not detected during the recovery from heat shock (39).

One of the most dramatic effects of heat shock is the repression of preexisting levels of protein synthesis. In *Drosophila* and human cells, repression of protein synthesis is accompanied by the rapid disaggregation of existing polysomes (31, 32). The fate of the mRNAs associated with polysomes before heat shock is not completely understood. These preexisting polysome-associated mRNAs have been shown to be associated with particles that sediment in the area of ribonucleoproteins or monosomes (33) or that are partially retained on polysomes but blocked in elongation (4). Heat shock has similar effects on protein synthesis in rabbit reticulocytes. The heat shock-induced disaggregation of polysomes and the repression of protein synthesis in rabbit reticulocytes or lysates are apparently due to the appearance of an inhibitor that affects formation of the initiation complex (36, 37). This inhibitor has been partially characterized and shares biochemical features with the heme regulatory inhibitor that appears in reticulocyte lysates that have not been supplemented with hemin (8). One proposed mechanism for repression of protein synthesis in rabbit reticulocytes involves the phosphorylation of the initiation factor eIF-2a (8). We presume that the mechanism for repression of globin synthesis in chicken reticulocytes is similar to what has been previously observed for rabbit reticulocytes. Furthermore, the efficient translation of HSP70 could reflect alternative pathways of protein synthesis whereby the efficient translation of HSP70 mRNA is not dependent on hemin control.

Upon return of heat-shocked *Drosophila* and chicken MSB cells to control temperatures a gradual recovery of normal patterns of protein synthesis occurs that is correlated with the repression of HSP70 synthesis (15; S. S. Banerji, L. Berg, and R. I. Morimoto, manuscript in preparation). We

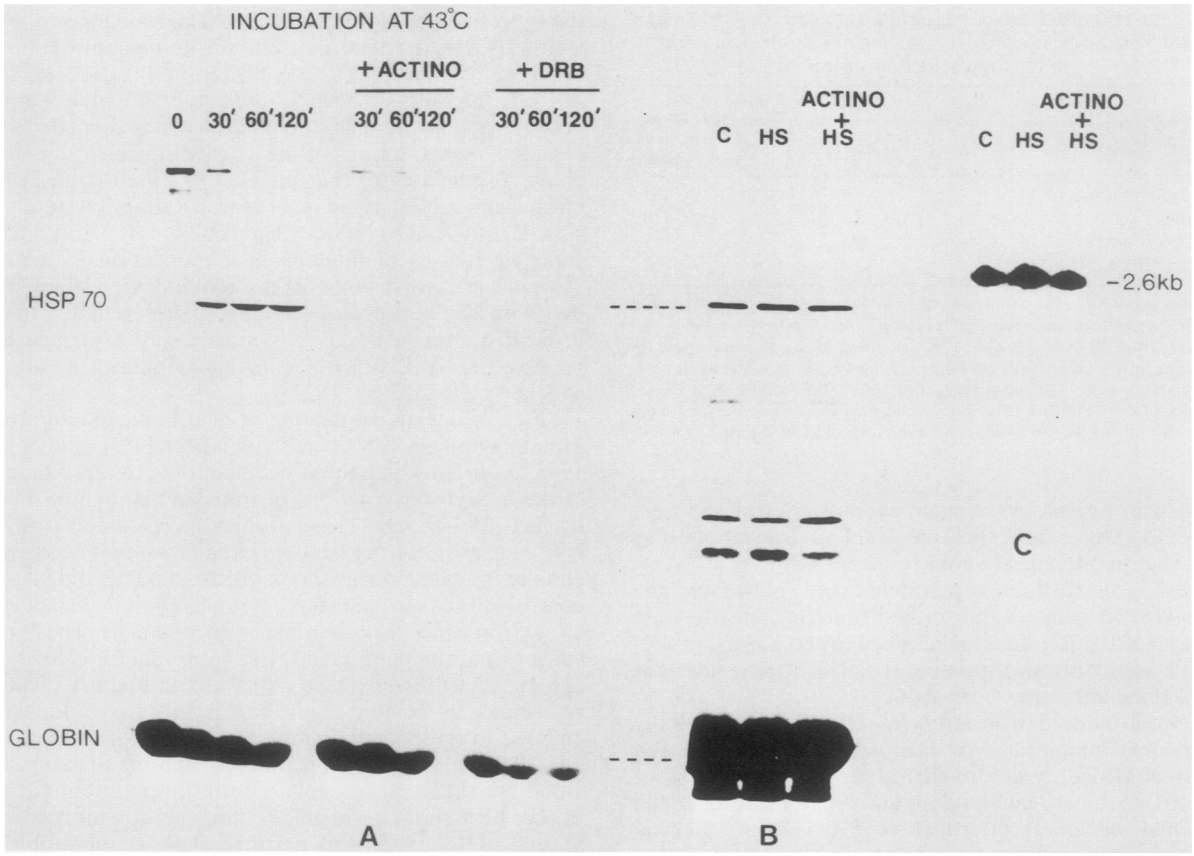

FIG. 8. Inhibition of preferential translation of HSP70 in heat-shocked reticulocytes by preincubation with the transcription inhibitors actinomycin D and DRB. (A) Reticulocytes were incubated at 43°C for 30, 60, or 120 min. A sample of cells was preincubated in the presence of 10 μg of actinomycin D per ml or 75 μM DRB for 20 min before incubation at the elevated temperature. At each time point a sample was obtained, and the cells were pelleted, suspended in 50 μCi of [^{35}S]methionine per ml, and incubated for 20 min at 37°C. The samples were lysed upon addition of SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. (B) Total poly(A)$^+$ mRNA prepared from control cells (C), heat-shocked (43°C) cells (HS), and cells pretreated with 10 μg of actinomycin D per ml for 30 min (ACTINO + HS) was in vitro translated in an RNA-dependent rabbit reticulocyte system. (C) Northern blot analysis of the same mRNA samples in (B) hybridized to ^{32}P-labeled pC1.8, 28-h exposure.

find that overall levels of protein synthesis, specifically globin synthesis, do not return to pre-heat shock levels if heat-shocked reticulocytes are returned to 37°C for periods of up to 6 h (our unpublished data). Our results with chicken reticulocytes suggest that the alterations in the protein-synthetic machinery initiated by heat shock are not reversed by returning cells to 37°C. The inability of reticulocytes to recover normal protein synthesis may be related to its unique developmental state because of reticulocytes are the preterminal differentiated state of erythropoiesis. The absence of recovery of protein synthesis in reticulocytes could suggest that the factor(s) necessary for reversal is not available in the heat-shocked reticulocyte. This conclusion is further supported by studies with rabbit reticulocytes that have demonstrated the inability of heat-shocked cells to recover normal levels of protein synthesis (36).

Our results with chicken reticulocytes show that continued transcription is required for the preferential translation of HSP70 in the heat-shocked cell despite preexisting levels of HSP70 mRNA. We suggest two possible explanations for this intriguing result. Perhaps only the newly transcribed HSP70 mRNA is preferentially translated. This mechanism would require the protein-synthetic machinery to discriminate between existing and newly transcribed mRNAs. Alternatively, transcription inhibitors could block the synthesis of a positive regulatory factor that is required for the efficient in vivo translation of HSP70 mRNA. This latter hypothesis is supported in part by McCormick and Penman (31), who demonstrated that pretreatment of HeLa cells with actinomycin D prevented the reformation of polysomes presumably involved in the translation of heat shock proteins. The possible requirement of an RNA-containing factor for the synthesis of heat shock proteins shares some similarities with the relationship between adenovirus VAI RNA and the translation of adenovirus late mRNAs (50).

Translational control of protein synthesis provides the cell with a powerful mechanism by which the translational apparatus can be rapidly reprogrammed to synthesize new or altered levels of specific proteins. Generally defined, translational control could operate on any of the components of the protein-synthetic apparatus including ribosomes, initiation and elongation factors, nucleotide cofactors, or at the structural level by recognition of the primary sequence or secondary structure in mRNA. Regulation of expression at the level of translation has been described for a variety of diverse systems including embryonic development in the surf clam *Spisula* (44), infection of human cells in culture with adenovirus (3, 5), and erythroid differentiation (23). The mecha-

nism of translational control of protein synthesis is complex due to the different levels of control. For example, there is substantial information to suggest that mRNA competition has a central role in viral expression and the takeover of the host translational apparatus (10, 42, 52). In adenovirus-infected cells host mRNA is stably maintained in an inaccessible complex in the cytoplasm (3).

We expect that the characterization of the heat shock-regulated translational control system in reticulocytes will provide access to biochemical analysis of the control elements involved in regulation.

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