In Vitro Synthesis of Heat-Shock Proteins by mRNAs from Chicken Embryo Fibroblasts*

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The pattern of proteins synthesized by chicken embryo fibroblasts changes dramatically after these cells are incubated at 45°C for a few hours. Three proteins (Mr = 22,000, 76,000, and 95,000) account for almost 50% of the cell's protein synthetic capacity immediately after the heat-shock (Kelley, P. M., and Schlesinger, N. J. (1978) Cell 15, 1277-1286). When mRNAs were isolated from heat-shocked cells and translated in a cell-free protein synthesizing system, a pattern of proteins virtually identical with that made by intact heat-shocked cells was detected. Mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radioimmuno precipitation with specific antisera were used to establish the identity of in vitro- and in vivo-generated heat-shock proteins. The mRNAs coding for the major heat-shock proteins could be separated by rate zonal centrifugation in a sucrose gradient and mRNAs with sedimentation coefficients of 20 S, 18 S, and 13 S were translated in vitro to yield proteins of 85, 76, and 22 kilodaltons, respectively.

We recently described a dramatic change that occurs in the pattern of protein synthesis when chicken embryo fibroblast cells are incubated for a few hours at 45°C (1). This brief heat shock did not inhibit general protein synthesis when cells were returned to their normal physiological temperature but these cells now diverted more than 50% of their protein synthetic capacity as a response to temperature stress. This response of avian cells to high temperatures closely parallels the effect of heat shock on cells of Drosophila melanogaster (reviewed in Ref. 2). An extensive amount of research has been devoted to studying the regulatory events following heat shock of the insect cells and there is clear evidence that new messenger RNA transcription occurs at a limited number of genetic loci immediately following the heat-shock. In the avian as well as in the insect system, the newly formed mRNAs appear to dominate the population of functional mRNAs in the cell and we might predict that in vitro translation of the mRNA pool from heat-shocked cells would show the preferential synthesis of the heat-shock proteins. To test this hypothesis, we prepared a cell-free protein synthesis system from mouse ascites cells and examined by SDS-polyacrylamide gel electrophoresis the polypeptides made when mRNA from normal and heat-shocked cells were added to these extracts. In this communication, we show that in vitro translation of mRNAs from heat-shocked chick cells produced a protein pattern that is virtually identical with that found in the intact heat-shocked cell. In addition, we were able to separate these mRNAs into specific size classes based on their sedimentation velocities in a sucrose gradient. Analyses similar to those described here have been reported for polysomal RNAs from the heat-shocked insect cells (3-6). Thus, this type of response to temperature stress appears to be strongly conserved in nature.

MATERIALS AND METHODS

Isolation of mRNA— Cultures of secondary chicken embryo fibroblast cells were grown to confluence in roller bottles with minimal Eagle's medium plus 3% fetal calf serum. After incubation for 4 h at either 37°C or 45°C, cells were washed three times with 0.1 M NaCl, 50 mM Tris/HCl (pH 7.5). Several of the culture bottles were labeled with 50 μCi each of [5-3H]uridine, (27.9 Ci/mmol, New England Nuclear) after the bottles had reached a temperature of 45°C. Cells were lysed with TNE buffer containing 1% SDS and scraped into a centrifuge bottle. RNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) twice and the aqueous layer was extracted with chloroform/isoamyl alcohol (24:1) twice with diethyl ether. RNA was precipitated by addition of 2½ volumes of ethanol and resuspended in TNE buffer containing 1% SDS. Polyadenylated mRNA was isolated on an oligo(T)-cellulose column (Collaborative Research, Waltham, MA) according to directions of the manufacturer. Fractions of poly(A) mRNA that were eluted from the column were brought to 0.1 M NaCl and precipitated with ethanol. This RNA was dissolved in TNE buffer and precipitated twice with ethanol. Preparations of mRNA were stored at −70°C in small volumes of H2O. Concentrations of RNA were estimated on the basis that 1 mg/ml has an absorbance of 20 at 260 nm.

In Vitro Protein Synthesis—The mouse ascites cell-free system has been described (7, 8). We included one modification to reduce the activity of endogenous mRNA. Microsomal nucleoplas was added to the extracts according to the procedure of Pabon and Jackson (9) except that the nuclelease and ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) were used prior to adding the "master mix" containing amino acids and the energy-generating system. In vitro translation was carried out at 30°C in 25-μl volumes containing 9 μCi of L-[35S]methionine (1000 Ci/mm, Amerham). In Vivo Labeling of Cells—Tissue culture cells were labeled with [35S]methionine for 1 h at 37°C and extracts were prepared according to the methods previously described (1).

Preparation of Antibodies—The p22 and p76 proteins were purified from large batches of CEF cells grown in roller bottles and heat-shocked for 4 h at 45°C. Sonicated extracts of these cells were passed over a Whatman DE52-cellulose column and protein was eluted with a 0 to 0.4 M salt gradient (details to be published elsewhere). Heat-shock proteins were assayed by autoradiography of slab gel electropherograms of samples from the column. Preparative slab gels were used to obtain homogeneous preparations. The p22 and p76 proteins were separated by rate zonal centrifugation in a sucrose gradient and mRNAs with sedimentation coefficients of 20 S, 18 S, and 13 S were translated in vitro to yield proteins of 85, 76, and 22 kilodaltons, respectively.
were eluted from these gels, dialyzed, lyophilized, and mixed in Freund's complete adjuvant. One milligram was injected into the footpads of a 6-month-old New Zealand white rabbit. Two weeks later, a booster of 500 µg of protein was injected. Serum was collected 1 week later.

Radioimmune Precipitation—Labeled tissue culture cells were washed twice with NaCl/P buffer and lysed with 0.5% Triton X-100 in NaCl/P buffer. The lysate was centrifuged 45 min at 100,000 × g in a Spinco 65 rotor at 4°C and the supernatant fractions were stored at −20°C. Samples from the in vitro reactions were centrifuged 15 min in a Beckman Airfuge at 30 p.s.i. and the supernatant fractions were processed immediately as follows. A 10% solution of Staphylococcus aureus, Cowan strain (10), was washed once with HNB buffer and suspended as a 10% solution in HNB buffer containing 0.02% sodium azide, 0.1% gelatin, and 1% Triton X-100. Samples of 35S-labeled protein were preabsorbed to the washed S. aureus in the presence of a preimmune rabbit sera (20 µl of serum/ml of sample and 400 µl of the 10% S. aureus). After a 5-min adsorption, the bacteria were removed by a 1-min centrifugation in an Eppendorf Microfuge. The supernatant fraction was distributed into four equal samples; one was used directly for analysis by polyacrylamide gel electrophoresis, and the remaining samples were combined with a preimmune sera, the anti-p22 sera, and the anti-p76 sera (5 µl of serum was used). After 30 min, the S. aureus solution was added for a further 10-min adsorption period. The samples were centrifuged as above and the pellets were washed twice with 50 mM Hepes/HCl (pH 7.4), 1.0 M NaCl, 0.1% gelatin and once with HNB buffer. Pellets were suspended in 50 µl of 5% β-mercaptoethanol, 2% SDS, 0.12 M Tris/HCl (pH 6.8), 10% glycerol, and the insoluble material was removed by centrifugation. The clear supernatant fraction was boiled for 5 min prior to analysis on a 12.5% polyacrylamide slab gel.

Sucrose Gradient Analysis of RNA—The pool of poly(A) mRNA (0.7 mg) isolated from heat-shocked cells was dissolved in 10 mM Hepes (pH 7.5), 1 mM EDTA, heated at 65°C for 10 min, cooled rapidly, and immediately layered on a 5 to 20% linear sucrose gradient containing the same buffer (11). Gradients were centrifuged in the Spinco SW 41 rotor at 41,000 rpm at 4°C for 12 h. Twenty-four fractions were collected and RNA in each fraction was precipitated with ethanol twice prior to adding to the in vitro translation system. The same volume of RNA from each fraction was used.

SDS Gel Analysis of Proteins—Proteins were separated on SDS-polyacrylamide slab gels according to the method of Laemmli (13). All samples were made 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.125 M Tris/HCl (pH 6.8), and boiled for 5 min prior to loading onto gels. Gels were dried and radioactive bands were detected by fluorography (13). All figures are fluorograms using Kodak X-omat R film.

RESULTS AND DISCUSSION

When mRNA from chick cells that had been incubated at 45°C for 4 h was added to an ascites cell-free protein synthesis system, a pattern of proteins was produced that closely resembled that made in intact heat-shocked cells (Fig. 1, Lanes 2 and 4). Three proteins with Mw = 95,000, 76,000, and 22,000 (p95, p76, p22) dominate the pattern of proteins synthesized after heat shock. These polypeptides were not made in large amounts in cells cultured at 37°C nor were they translated in large amounts in vitro by RNAs from cells grown at 37°C (Fig. 1, Lanes 1 and 3). The major protein synthesized by non-heat-shocked cells in vivo and in vitro was actin, with Mc = 42,000. Very low amounts of protein were made by the endogenous mRNA present in the nuclease-treated ascites cell extracts (Fig. 1, Lane 5).

The three major heat-shock proteins made in vitro have electrophoretic mobilities in SDS-polyacrylamide gels that were indistinguishable from those detected in vivo. In addition, antisera raised in rabbits against purified preparations of p22 and p76 specifically precipitated proteins of these molecular weights from in vitro reaction mixtures that contained mRNA from heat-shocked cells (Fig. 2A, Lanes 7 and 8). The anti-p76 sera showed a strong reaction against the p76 protein made in vivo by heat-shocked cells and also precipitated small amounts of two proteins of this molecular weight present in extracts of cells grown at 37°C and synthesized in vitro by extracts incubated with mRNA prepared from 37°C CEF cells (Fig. 2B, Lanes 4 and 8). No significant amount of 35S-labeled proteins were made in vivo or in vitro at 37°C that were recognized specifically by the anti-p22 sera (Fig. 2B, Lanes 3 and 7). However, this antiserum precipitated a small amount of two additional proteins (Mc = 31,000 and 17,000) made in vitro in extracts incubated with mRNA from the heat-shocked cells (Fig. 2A, Lane 7). The relationship of these proteins to p22 is under investigation.

The sizes of the mRNAs coding for the heat-shock proteins was obtained by centrifuging the pool of mRNA from heat-shocked cells through a 5 to 20% sucrose gradient and analyzing each fraction for its in vitro translation product (Fig. 3A). We found that the major heat-shock proteins were translated by mRNAs with sedimentation coefficients of 13 to 35 kilodaltons. A protein of this size was detected in vivo in cells incubated at 37°C; Lanes 2 and 4 are from cells incubated at 45°C. Experimental details are described under "Materials and Methods." For in vitro reactions, 3 µg of mRNA were used and incubations were performed at 30°C for 60 min. Lane 5 was not supplemented with exogenous mRNA. For the in vivo reactions of Lane 2, cells were treated at 45°C for 4 h before the 1-h labeling at 37°C. The separating gel contains 11.5% acrylamide.

FIG. 1. SDS-polyacrylamide slab gel pattern of 35S-labeled proteins synthesized in vivo and in vitro. Lanes 1 and 3 are from cells incubated at 37°C; Lanes 2 and 4 are from cells incubated at 45°C. Experimental details are described under "Materials and Methods." For in vitro reactions, 3 µg of mRNA were used and incubations were performed at 30°C for 60 min. Lane 5 was not supplemented with exogenous mRNA. For the in vivo reactions of Lane 2, cells were treated at 45°C for 4 h before the 1-h labeling at 37°C. The separating gel contains 11.5% acrylamide.
p95. Possibly, the 45-kilodalton protein was synthesized initially as a higher molecular weight precursor form.

These data show that there is remarkable fidelity of the mRNA pool obtained from heat-shocked avian tissue culture cells to reproduce in vitro those same proteins whose synthesis is greatly elevated in the intact heat-shocked cell. In addition, these mRNAs can be separated by sucrose gradient centrifugation and this procedure should allow for the preparation of appropriate cDNA probes that will enable us to measure changes in the amounts of these mRNAs during the cell's response to and recovery from heat shock. There is already evidence from our studies with the avian cell (1) and from the much more detailed analyses of the heat-shock Drosophila cell system (2) that new synthesis of RNA coding for the limited set of heat-shock protein begins very soon after the cells are brought to the higher temperature. This RNA appears in the cell's cytoplasm and effectively outcompetes the normal set of cellular mRNAs for the cell's protein synthesis machinery. The selective translation of heat-shock mRNAs could be accounted for by an increase in mass of these mRNAs relative to the normal set of messengers but there is also the possibility that heat-shock mRNAs are qualitatively different to the extent that they have greater affinity for ribosomes and initiation factors than do the normal cell messengers. With suitable cDNA probes, we should be able to quantitate the relative amounts of these heat-shock mRNAs.

The regulatory mechanisms operating in these heat-shocked cells is a most intriguing and considerable information is being accumulated about the organization and nucleotide sequence composition of the genetic elements that become activated in the heat-shocked Drosophila cells (2). It is also of considerable interest to determine the function of these few proteins whose synthesis dominates the cells' synthetic machinery. Heat shock has been studied in a variety of species including bacteria, insects, birds, and mammals (1, 2, 14, 15) and there is a striking similarity in the number and sizes of the proteins included in these widely diverse organisms.

**Fig. 2.** SDS-polyacrylamide slab gel patterns of 35S-labeled proteins precipitated with antisera against p22 and p76. Lanes 1 and 5 are extracts used for the radioimmune precipitation (see "Materials and Methods"); Lanes 2 and 6 are from reactions with preimmune sera; Lanes 3 and 7 are from reactions with anti-p22 sera; Lanes 4 and 8 are from reactions with anti-p76 sera.

**Fig. 3.** Analysis of mRNA after rate zonal sedimentation in a sucrose gradient. A, SDS-polyacrylamide gel pattern of in vitro reaction products formed by mRNAs present in the gradient fractions noted above the lanes; fractions are numbered from the bottom of the gradient. A 10% acrylamide gel was used. B, plots of the relative amounts of the 22-kilodalton protein (●), the 76-kilodalton protein (○), and the 95-kilodalton protein (▲) formed by mRNA in the different gradient fractions. Fluorograms were analyzed by densitometric tracing in a Gilford spectrophotometer equipped with a linear transport scanner device.
In no case, however, has there been a biological or enzymatic function associated with a heat-shock protein. Some of the heat-shock proteins are found in cells that have been subjected to other kinds of stress; the review by Ashburner and Bonner (2) lists 30 materials or treatments that can induce heat-shock proteins. In addition, several chelating drugs, sulfhydryl reagents, and transition series metals have been shown to induce in CEF a set of proteins which include the heat shock proteins (Refs. 16 and 17; Footnote 2). Most of these treatments can affect the cell's state of oxidation and there has been speculation and some suggestive evidence that dehydrogenases are among those proteins induced by these treatments. For example, synthesis of the alcohol dehydrogenase in maize seedlings was induced to high levels after anoxia (18). This enzyme is a terminal dehydrogenase in the plant glycolytic pathway and it may be that other dehydrogenases as well as central controlling enzymes of the glycolytic pathway are induced in the heat-shocked avian cells. Antibodies directed against the avian heat-shock proteins should prove valuable in determining the precise enzymes induced by heat shock, for our preliminary data indicate that these proteins are present, albeit synthesized in lower levels, during normal cell growth.

REFERENCES