hsp108, a Novel Heat Shock Inducible Protein of Chicken†

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Received February 20, 1986; Revised Manuscript Received May 13, 1986

ABSTRACT: cDNA clones encoding a protein that copurifies with the progesterone receptor B subunit but does not bind progesterone have been described [Kulomaa, M. S., Weigel, N. L., Kleinsek, D. A., Beattie, W. G., Conneely, O. M., March, C., Zarucki-Schulz, T., Schrader, W. T., & O'Malley, B. W. (1986) *Biochemistry* (preceding paper in this issue)]. A full-length sequence for these clones was derived and was found to encode a protein that is structurally unrelated to the progesterone receptor but that contains significant homologies to the previously described heat shock proteins hsp90 of yeast and hsp83a of *Drosophila melanogaster*. In this paper it is shown that this protein is indeed a heat shock protein. Though the apparent molecular weight of the protein is 108 000 on sodium dodecyl sulfate–polyacrylamide gels, the molecular weight of the polypeptide backbone is 92 000. The steady-state level of gene transcripts as well as the level of protein is inducible by heat shock, but the gene is constitutively expressed in a number of tissues. A previously undescribed heat shock protein of molecular weight 78 000 in these preparations is also reported.

A common feature of the response of eukaryotic and prokaryotic organisms to environmental stress is the rapid production of a defined set of proteins, the heat shock or stress proteins [for reviews see Nover (1984), Schlesinger et al. (1982), and Neidhardt et al. (1984)]. As of yet the mode of function of these proteins is not understood, though their presence correlates with increased thermal tolerance in the organism. Throughout the eukaryotes these proteins appear to be highly conserved. They have been most extensively studied in *Drosophila melanogaster* where eight major heat shock proteins have been identified, the largest of which has an apparent molecular weight of 83 000 as measured by SDS–polyacrylamide gel electrophoresis.[1] In HeLa cells, no 83 000 molecular weight heat shock protein is found, but a number of other high molecular weight proteins have been shown to be inducible by heat shock [apparent molecular weight 85 000–90 000, 100 000, and 110 000 (Thomas et al., 1982)]. Similar proteins have been identified in other mammalian cell lines (Landry et al., 1982; Subjeck et al., 1982). In the chicken, four heat shock proteins have previously been identified. These have apparent molecular weights of 90 000, 70 000, 24 000, and 8000 (Kelley & Schlesinger, 1978; Bag, 1983; Bond & Schlesinger, 1985). The gene for the last of these has been cloned and was shown to encode ubiquitin (Bond & Schlesinger, 1985). Very recently a short cDNA segment for the gene encoding the 90 000 molecular weight protein also has been cloned (Catelli et al., 1985).

Recently we have cloned a gene encoding a protein that copurifies but is not physically associated with the progesterone receptor B subunit (Zarucki-Schulz et al., 1984). This protein has an apparent molecular weight on SDS–polyacrylamide gels of 108 000 and has certain biochemical similarities to the receptor but will not bind progesterone. Furthermore, it can be clearly distinguished now as a protein that is structurally unrelated to the authentic progesterone receptor B. We have now derived and sequenced the full-length cDNA and genomic clones of the "B antigen" (Kulomaa et al., preceding paper; Kleinsek et al., unpublished data). Computer analysis of the DNA sequence of the gene revealed homologies with two previously identified heat shock proteins, yeast hsp90 (Farrelly & Finkelstein, 1984) and *Drosophila* hsp83a (Holmgren et al., 1979).

We have previously shown that the antigen mRNA is steroid inducible (Zarucki-Schulz et al., 1984; Baez et al., unpublished data). Here we show that the antigen is indeed a heat shock inducible protein not previously identified in the chicken. The homology to the yeast and *Drosophila* proteins is maintained throughout the length of these proteins, but the chicken protein has an amino-terminal extension when compared with these proteins. We have now named the protein hsp108 after its apparent molecular weight on SDS–polyacrylamide gels and in keeping with previous published data on this protein. However, the molecular weight of the polypeptide backbone as calculated from its sequence is 91 555. Induction by heat stress occurs both at the RNA and protein levels, but the protein is constitutively expressed in many chicken tissues.

Experimental Procedures

Materials. [^{32}P]GTP (3000 Ci/mmol) and [^{35}S]methionine (1000 Ci/mmol) were from Amersham. Other radionucleotides were from ICN. DNA polymerase I (Kornberg enzyme) was from Boehringer Mannheim. Rabbit anti-rat IgG was from Zymed. Other chemicals and enzymes were analytical reagent grade.

Amino Acid Sequence of hsp108. Full-length cDNA and genomic clones of hsp108 have been derived and completely sequenced (Kulomaa et al., 1986; Kleinsek et al., unpublished data). Amino acid sequence derived from these clones was analyzed by using the Beckman "Microgenie" library of routines.

Primary Cell Cultures and Heat Shock. Primary chicken oviduct cell cultures were prepared from diethylstilbestrol (DES) stimulated 5-week-old white Leghorn chicks as previously described (Dean et al., 1983) and grown in Dulbecco's minimal essential medium (DMEM) supplemented with 5% horse serum and 10^{-7} M DES. Cultures were withdrawn from DES stimulation by growth in DMEM medium, supplemented

† This work was supported by National Institutes of Health Grants HD08188 and HD07495 to B.W.O. and HD17379 to M.-J.T.
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[1] Abbreviations: DES, diethylstilbestrol; DMEM, Dulbecco's minimal essential medium; EDTA, ethylenediaminetetraacetate; hsp108, chicken 108-kDa heat shock protein; IgG, immunoglobulin G; kDa, kilodalton; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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with charcoal-stripped horse serum, for periods stated in the text. Primary chicken myoblast cultures were prepared from 11-day embryos by the method of Fishbach (1972). They were allowed to differentiate for 3 days prior to heat shock. T47D and chicken bursal lymphoma cells (line S-13) were maintained as described elsewhere (Horowitz et al., 1982). All cell cultures were maintained at 37 °C in 10 mL of medium in 10-cm culture dishes. To heat shock cells, they were moved to a 45 °C incubator for the times stated. They were then harvested or allowed to recover at 37 °C for the times stated.

Hormone Treatment of Animals. Two-week-old white Leghorn chicks were injected daily for 12 days with 2 mg of DES in 0.2 mL of sesame oil. They were subsequently withdrawn from hormone for 5 days. Sixteen hours after a single further injection of DES, chicks were sacrificed and tissues taken for analysis.

Isolation of Total Cellular RNA. RNA from tissue-culture cells was prepared by a modification of the method of Chirgwin et al. (1979). Cells on plates were dissolved in 2.5 mL/plate of guanidine thiocyanate buffer [5 M guanidine thiocyanate (Fluka), 0.025 M sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% sodium *N*-lauroylsarcosinate, and 0.1% Sigma 30% Antifoam A (v/v)]. The solution was layered over 1.5 mL of cesium chloride buffer (5.7 M cesium chloride, 0.025 M sodium acetate, pH 5.0, and 0.002 M NaEDTA) in a polyallomer tube and centrifuged for 16 h at 20 °C and 76000*g*. Pellets were resuspended in 300 μL of 0.1% SDS, 0.3 M sodium acetate, pH 5.2, and 10 mM NaEDTA and extracted with an equal volume of buffer-saturated phenol, pH 5. After extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and with chloroform/isoamyl alcohol (24:1), the aqueous phase was precipitated with 2.5 volumes of ethanol. The RNA pellet was resuspended in sterile water prior to use.

Probes and RNA Blot Hybridization Analysis. Concentrations of various gene transcripts in total RNA were determined by RNA capillary blot hybridization ("Northern") analyses. Usually 15 μg of total RNA was fractionated by electrophoresis in a 1.2% agarose gel containing 2.2 M (6.7% v/v) formaldehyde and 20 mL sodium phosphate, as previously described (Lai et al., 1983). RNA was blotted onto nitrocellulose filters and immobilized by baking for 2 h at 80 °C. Filters were prehybridized for at least 4 h in hybridization buffer [50% v/v formamide, 5 × SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 0.05 M sodium phosphate, pH 7.0, 100 μg/mL yeast tRNA, 0.25% nonfat dried milk (Johnson et al., 1984)] at 42 °C. Hybridization with the appropriate nick-translated probes [(2–3) × 10^8 cpm/μg] was for at least 16 h in the same conditions. Filters were washed to a stringency of 0.1 × SSC and 0.1% SDS at 52 °C prior to autoradiography. Probes were prepared from the following plasmids: for hsp108 RNA, pPRB-6 (Zarucki-Schulz et al., 1984) and pHSP2.8 [a genomic 3′ end clone containing no structural sequences (Kleinsek, unpublished results)]; for glyceraldehyde-3-phosphate dehydrogenase, pGAD-28 (Dugaiczyk et al., 1983).

Cytosolic Extracts and Immunodots. Cell culture or tissue samples (50–100 mg net weight) were disrupted by sonification (2 × 20 s, Heat System sonifier) in 2 mL of protein sample buffer (0.15 M NaCl, 0.05 M Tris–HCl, pH 7.5, 2 mM dithiothreitol, 5 mM EDTA, 0.2% Tween-20). Cell debris was removed by low-speed centrifugation and protein concentration measured by the method of Bradford (1976). For immunoelectroblots ("Western blots"), 20-μg protein samples were fractionated by electrophoresis on 7.5% polyacrylamide gels containing 0.1% SDS according to the method of Laemmli (1970). Electroblotting and immunoautoradiography were performed essentially as previously described (Edwards et al., 1984), with the modifications of Johnson et al. (1984). For protein immunodots, serial dilutions of the tissue and cell extracts were prepared and were brought to 40 μg of total protein with bovine serum albumin. Protein dots were made on nitrocellulose with a Bio-Rad "minifold" apparatus, and probed with 9G10 antibody, just as for the electroblots.

Pulse Labeling of Cellular Proteins and Immunoprecipitations. Proteins from chicken primary oviduct cell cultures in 10-cm Petri dishes were labeled with [^{35}S]methionine. Cells grown in DMEM supplemented with 5% charcoal-stripped horse serum were subjected to heat shocks as stated (see figure legends). They were then washed with incomplete DMEM, without methionine, and 3 mL of labeling medium [DMEM–methionine, supplemented with 2% dialyzed, charcoal-stripped, fetal calf serum and 15 μCi of [^{35}S]methionine (500 Ci/mmol)] was added to each plate. Cells were labeled for 1 h at 37 °C. Cells were then harvested with a rubber policeman, washed in Hank's medium without Ca^{2+} and Mg^{2+} (Hanks, 1949) and frozen in liquid nitrogen. Cell pellets were stored at −70 °C until used to prepare whole cell sonicates as before.

Proteins from ^{35}S-pulse-labeled cells were immunoprecipitated with monoclonal antibody 9G10. Usually 100 000 cpm of pulse-labeled proteins (50–150 μg of total protein) were shaken overnight at 4 °C with 8 μg of purified 9G10 antibody in a total volume of 200 μL of protein sample buffer (as before). Thirty micrograms of rabbit anti-rat IgG antibody was added, and reactions continued for 4 h at 4 °C. Subsequently 25 μL of protein A–Sepharose was added, and reactions continued for a further 2 h. The immunoprecipitate was collected by contrifugation, washed 5 times in protein sample buffer, and analyzed by electrophoresis on SDS–7.5% polyacrylamide gels as before.

RESULTS

Chicken hsp108 Is Homologous to Yeast and Chicken hsp90's and to D. melanogaster hsp83a. When the full cDNA sequence (Kulomaa et al., 1986) of hsp108 was compared with other published DNA sequences by using the NIH Gen Bank, homologies with two other eukaryotic proteins, yeast hsp90 (Farrelly & Finkelstein, 1984) and *Drosophila* hsp83a (Holmgren et al., 1979), were revealed. These are displayed in Figure 1, together with a homology with a recently sequenced segment of a chicken gene, hsp90 (Catelli et al., 1985). The overall homology between hsp108 and yeast hsp90 (the two full-length sequences) is 49.4% at the amino acid level, and conserved regions are maintained throughout the length of the shorter (yeast) gene. However, the homology between yeast and *Drosophila* genes is somewhat higher in the published region (64.4%), and the chicken hsp90 gene is 82.5% identical with the yeast and 90% identical with the *Drosophila* genes over the 40 amino acids of published sequence but only 48.8% identical with hsp108 in this region. hsp108 and hsp90 therefore define a group of at lease two related genes in the chicken. The other identified member of the group already has been shown to be a heat shock protein.

The difference in apparent molecular weight between hsp108 and yeast hsp90 is due in part to a 71 amino acid amino-terminal extension on the former. This includes a possible signal peptide sequence (underlined in Figure 1). No similar sequence is found in the yeast or *Drosophila* genes. In other respects, predictions of secondary structure (Chou & Fasman, 1974) and hydropathy profile (Kyte & Doolittle, 1982) for yeast hsp90 and chicken hsp108 show them to be rather similar (data not shown). We therefore did experiments to show

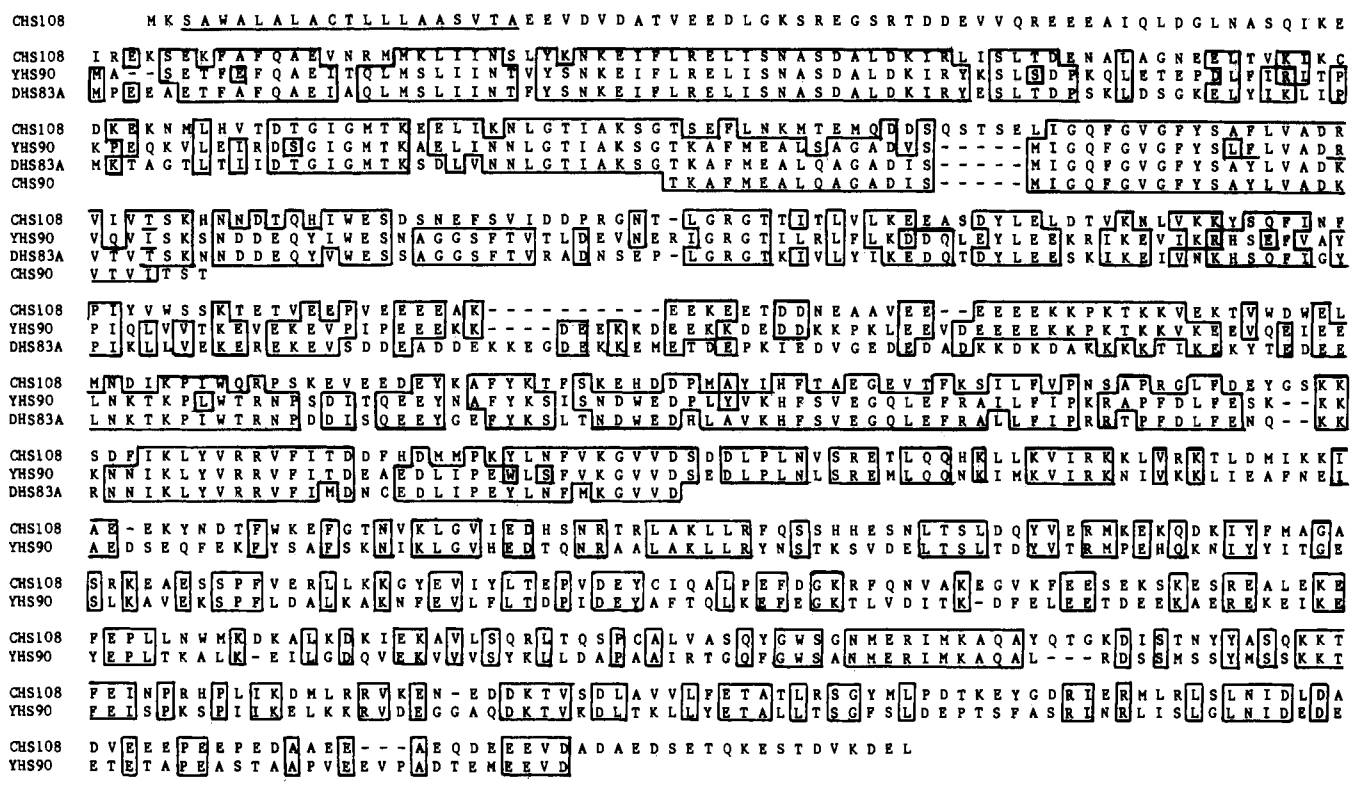

FIGURE 1: Comparison of chicken hsp108 with yeast hsp90, *D. melanogaster* hsp83a, and chicken hsp90. The amino acid sequence was derived from the cloned sequences of each gene and has been aligned to maximize areas of homology. The sequence of the *D. melanogaster* hsp83a does not include the carboxyl terminal of the molecule, which has not been published, and only a 40 amino acid segment of chicken hsp90 has been sequenced. A possible signal peptide in chicken hsp108 is underlined. CHS108, chicken hsp108; YHS90, yeast hsp90 (Farrelly & Finkelstein, 1984); DHS83A, *Drosophila* hsp83a (Holmgren et al., 1979); CHS90, chicken hsp90 (Catelli et al., 1985).

whether hsp108 was indeed a heat shock gene.

hsp108 Is Expressed Constitutively in Many Tissues. We examined the tissue distribution of hsp108 in the 5-week-old chicks, using the monoclonal antibody 9G10 in "Western" immunoblots. Figure 2A shows that the protein was detected in all tissues examined, with the exception of skeletal muscle. The abundance of the protein varies with the tissue examined, but in all cases it is in the region of 0.05–0.5% of total protein. The experiment also shows the presence of an antigenic protein of the same molecular weight in chicken bursal lymphoma cells and in T47D cells (a human breast carcinoma cell line). We also have detected this antigen in cell lines and tissues from rabbit, mouse, monkey, and *Xenopus* (data not shown), suggesting it is widespread and highly conserved across vertebrate species.

We used a second approach to examine distribution of the antigen in the estrogen-stimulated chick (Figure 2B). Fifteen micrograms of total RNA from each tissue was fractionated on agarose gels and transferred to nitrocellulose filters. It was probed with a segment of the cloned cDNA coding for hsp108 (Zarucki-Schulz et al., 1984). We have previously shown an mRNA of 2750 bases hybridizes to this cDNA segment (Zarucki-Schulz et al., 1984). Once again, all examined tissues contained mRNA for hsp108, but the level found in blood was much lower than that found elsewhere. Similarly, hsp108 mRNA was also found in various tissues obtained from hen and rooster (data not shown). In these blots, minor signals are seen at 3500 and 4400 bases. We believe that these are due to nuclear precursors of hsp108 and not to related gene products (data not shown).

hsp108 Is Inducible by Heat Shock in Primary Cell Cultures. Figure 2A shows that the 9G10 antibody is exremely specific in its reaction to hsp108 in a mixture of proteins immobilized on nitrocellulose. This protein has an apparent molecular weight of 108 000. We have therefore designated this protein hsp108. Because of this specificity we were able to use a "protein dot" type immunoblot assay to measure the relative concentration of hsp108 in various protein samples. Initially, we performed heat shocks on primary cultures of chicken oviduct cells (Dean et al., 1983), because we have already shown that transcription of the gene is steroid inducible (Zarucki-Schulz et al., 1984; Baez et al., unpublished results), and these cells retain steroid receptors. We were interested in the interaction between steroidal and heat shock control responses of the gene. Cells were cultured at 37 °C and shifted to 45 °C for periods of $^1/_2$–3 h. However, we were unable to demonstrate reproducible increases in the total concentration of hsp108 in these cells inder these conditions (data not shown). The concentration of antigen even in control cells is very high, approaching 1% of total cell protein (possibly because of steroid induction of the protein during oviduct differentiation and subsequent plating of the cells). It is likely that any extra synthesis occurring in these cells during heat shock would not be seen in this assay because of the high preexisting levels of the gene product. A similar low level of induction was also observed in the T47D cells and primary myoblasts from 11-day chick embryos (data not shown).

To demonstrate that synthesis of hsp108 is inducible in oviduct cells, we used [^{35}S]methionine to label newly synthesized proteins in the cells before and after heat shocks administered as before. Three hours of heat shock caused the appearance of several prominent newly synthesized proteins in addition to those seen in control conditions (Figure 3A). As well as proteins of 72 000 and 90 000 apparent molecular weight, which probably correspond with previously identified chicken heat shock proteins, inducible proteins of 78 000 and 108 000 (molecular weights not corresponding to previously identified hsp's in the chicken) were observed. When aliquots

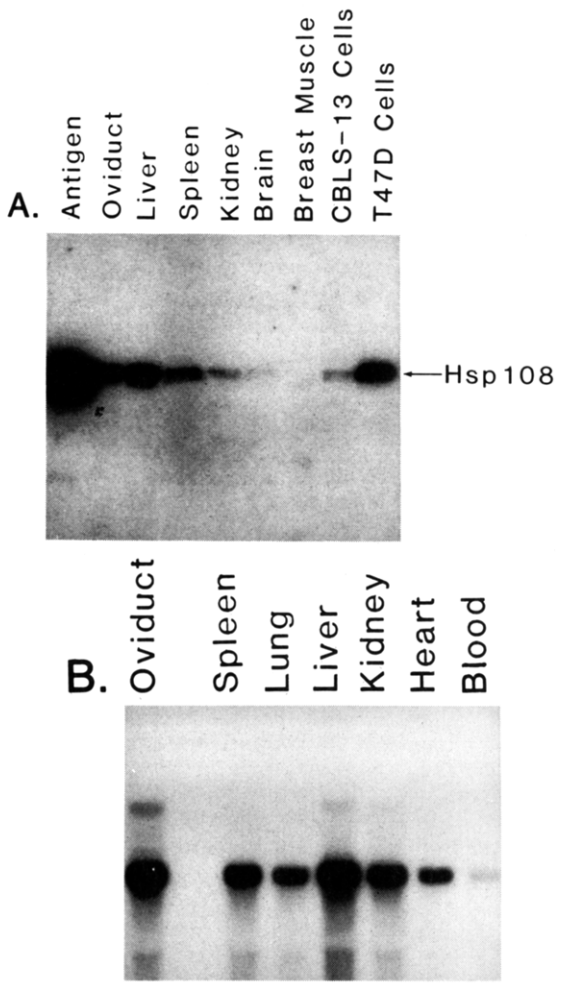

FIGURE 2: Tissue distribution of hsp108. (A) Immunoblot of hsp108 in various tissues. Protein extracts from various tissues of 5-week chicks subjected to stimulation with DES for 3 weeks or from two cell types were prepared as stated. Total protein (20 μg) in each tissue or cell type was subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose. The filter was then probed with monoclonal antibody 9G10, rabbit anti–rat IgG, and iodinated protein A. The lane labeled antigen contains 100 ng of purified hsp108 (Edwards et al., 1984). (B) Total RNA (15 μg) was prepared from various tissues of chronically DES stimulated chicks. After fractionation by electrophoresis and transfer to nitrocellulose filters as stated, it was probed by hydridization to the hsp108 cDNA clone pRB-6 (Zarucki-Schulz et al., 1984).

of these preparations were immunoprecipitated with the antibody 9G10 (Figure 3B), a precipitated protein with apparent molecular weight of 108 000 was heat shock inducible. Though a number of other proteins were present in these immunoprecipitates, none of them was heat shock inducible, and none reacted with the primary antibody, since addition of purified unlabeled antigen to the immunoprecipitates caused specific loss of signal from the 108 000 molecular weight band (Figure 3C). Thus hsp108 is also heat shock inducible in primary oviduct cell cultures.

Heat Shock Increases the Concentration of hsp108 Gene Transcripts. The relative concentration of hsp108 transcripts in total RNA from chicken primary oviduct cells that had been heat shocked for different lengths of time was measured. RNA samples were fractionated on agarose/formaldehyde gels, transferred to nitrocellulose filters and probed by hybridization with a 1.8-kb fragment from the cloned gene (PRB-6; Zarucki-Schulz et al., 1984). Figure 4A shows that the amount of probe hybridizing to RNA increases with time of heat shock to a maximum at ~3 h of heat shock. The degree of induction was from 5-fold to 8-fold, depending on the experiment, as measured by cutting out and scintillation counting the hybridized probes on the filter. In control experiments a glyceraldehyde-3-phosphate dehydrogenase probe was hybridized to the same filters, showing no substantial changes in the concentration of this RNA on heat shock.

We also measured the kinetics of recovery from heat shock and the accumulation of RNA during longer periods of heat shock, using the same techniques (Figure 4B). When cells are returned to 37 °C after a 3-h period of heat shock, during which maximal induction of hsp108 mRNA occurs, the level of hsp108 mRNA remains high for a further 3 h before declining to background levels over a period of 16 h. Surprisingly, similar kinetics are seen if heat shocks are simply extended over a period of 7.5 or 16 h. hsp108 mRNA levels are maximal from 3 to 6 h into heat shock and then decline, reaching control levels by 16 h of heat shock. A similar result was obtained when we used a genomic probe, hsp2.8, which contains only 3′ untranslated and flanking sequences (Figure 4C). Thus, probes from different regions of the hsp108 gene detected a similar response upon heat shock treatment. In addition, the glyceraldehyde-3-phosphate dehydrogenase control shows that this is not due to a generalized degradation of RNA at the long-time points. Furthermore, if cells are heat shocked for just 30 or 60 min and allowed to recover, hsp108 mRNA continues to accumulate for the first 3 h of the recovery period before declining (data not shown). It therefore appears that heat shock triggers a programmed expression of hsp108 that is independent of the length of the stress.

Discussion

We have shown that hsp108 is a newly described heat shock protein of chicken. The rate of protein synthesis in any tissue and at any time in heat shock is in close agreement with the steady-state level of RNA in the tissue, indicating that control of the heat shock response occurs prior to translation.

hsp108 is homologous to the yeast protein hsp90 and the *Drosophila* protein hsp83a. The latter has been shown to be constitutively expressed in many adult tissues and in many developmental stages (Zimmerman et al., 1983; Mirault et al., 1977; O'Connor & Lis, 1981). Expression is increased 6–10-fold by heat shock (O'Connor & Lis, 1981). These characteristics of the protein are very similar to hsp108 and also to the related chicken hsp90. In HeLa cells, heat shock proteins of 110 kDa, 100 kDa and 90 kDa have been identified (Thomas et al., 1982; Welch et al., 1982). The 100- and 90-kDa proteins display similar chromatographic properties and are both phosphoproteins. The amino acid composition of the 90-kDa protein is very similar to chicken hsp108 and yeast hsp90 (Welch et al., 1982). The amino acid composition of hsp100 and hsp110 has not been determined, but hsp100 is an acidic phosphoprotein like our hsp108 but unlike hsp110 (Welch et al., 1982). hsp100 is found associated with the Golgi apparatus of unshocked cells (Lin et al., 1982).

In unshocked *Drosophila* cells, hsp83a mRNA is not polyadenylated. It becomes polyadenylated on heat shock (Starti et al., 1980). In contrast, in chicken primary oviduct cells, hsp108 mRNA is polyadenylated even prior to heat shock (data not shown). Chicken hsp90 mRNA is more evenly divided between polyadenylated and nonpolyadenylated fractions (Catelli et al., 1985), but the degree of polyadenylation does not change upon heat shock. Although neither Catelli et al. (1985) nor ourselves (Kleinsek et al., unpublished results) have been able to find any indication of more than one gene for our respective heat shock proteins on "Southern" analysis, it seemed possible that the two genes

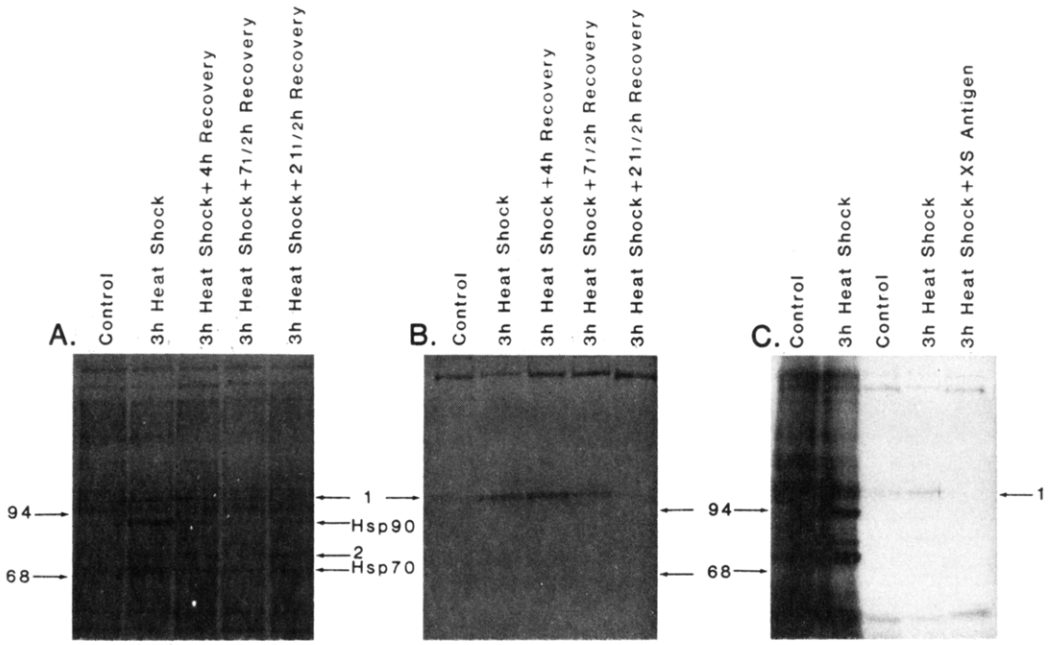

FIGURE 3: Pulse labeling of proteins in chicken primary oviduct cells with [^{35}S]methionine. Oviduct cells withdrawn from DES were grown at 37 °C or heat shocked and allowed to recover as before. At the times shown, total proteins (40 000 cpm) (A) or proteins immunoprecipitated with monoclonal antibody 9G10 (100 000 cpm) (B) were fractionated by electrophoresis on SDS–acrylamide gels and analyzed by fluorography. Positions of marker proteins are shown. (C) shows a control for the immunoprecipitation reaction. In the first two lanes, 40 000 cpm of control and heat shocked total protein was analyzed as before. In the next two lanes, 100 000 cpm of immunoprecipitated protein from the same samples was analyzed. In the final lane, 100 000 cpm of heat shocked protein was immunoprecipitated in the presence of 1.5 μg of purified hsp108. It is clear that the only heat shock responsive band in the immunoprecipitates is the protein with which 9G10 reacts. Arrows labeled 1 and 2 are the newly identified hsp108 (1) and hsp78 (2). Positions of previously identified proteins hsp90 and hsp70 also are shown, together with the positions of 68 000 and 94 000 molecular weight marker proteins (labeled 68 and 94).

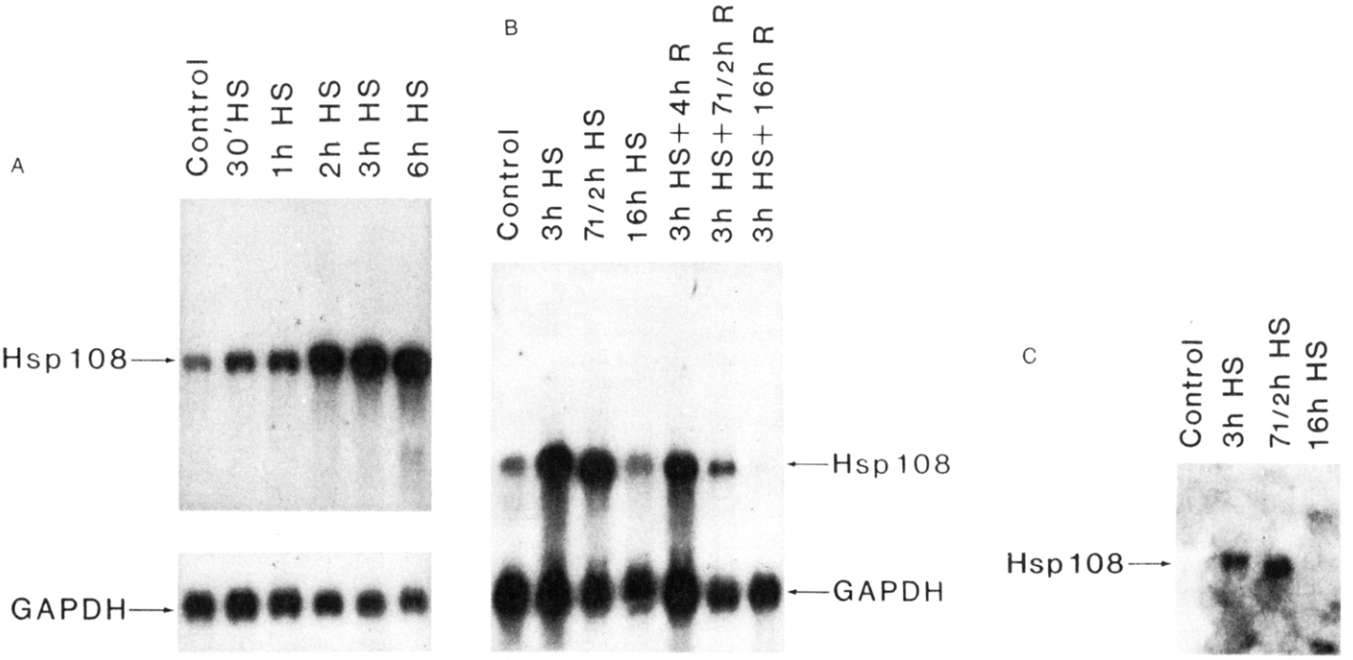

FIGURE 4: Quantitation of hsp108 RNA in primary oviduct cells during and after heat shock. (A) RNA was prepared from primary oviduct cells immediately after heat shocks of various lengths as shown. Northern blots were prepared as before and hybridized to the hsp108 cDNA clone, pPRB-6. The same filter was then rehybridized with pGAD-28, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) clone as a control for loading and transfer. (B) primary oviduct cells were given 3-h or longer heat shocks as stated and allowed to recover at 37 °C for the period shown. RNA was probed for hsp108 and glyceraldehyde-3-phosphate dehydrogenase transcripts with a mixture of pPRB-6 and pGAD-28 as a probe. Periods of heat shock at 45 °C (HS) and of subsequent recovery at 37 °C (R) are shown in hours. (C) Same as (B) except nick-translated genomic DNA clone hsp2.8 (containing 3′ untranslated and flanking sequences) was used as a probe.

could cross-hybridize at the RNA level. A short area of 90% homology does exist within both genes in the published sequence, and other such areas might allow cross-hybridization. The two RNA's are of very similar sizes, so such cross-hybridization would not be detected in our analysis. We cannot eliminate this possibility until the whole of chicken hsp90 has been cloned and we have attempted to cross-hybridize it with our gene. However, we have used a 3′ end clone of hsp108, containing no structural sequence, as a probe and observed induction kinetics identical with those seen when structural sequence is used. As this clone would be expected to have diverged much further from the hsp90 sequence than structural

sequences of hsp108, it is very unlikely that the inductions seen at the RNA level are caused by cross-hybridization to hsp90. In any event, hsp90 is clearly visible in our protein gels and is distinct from hsp108. We have previously shown that hsp90 has a different tryptic digestion pattern from hsp108 (Birnbaumer et al., 1984). However, the relationship between chicken hsp90 and hsp108 suggests that they may have evolved from a single ancestral gene to take specialized roles in the cell.

The induction kinetics of hsp108 are relatively slow for a heat shock protein. In *Drosophila*, heat shock proteins are switched on within 6–10 min, and maximal induction occurs within 30 min. However, the rates seen here are more typical of higher eukaryotes. Chinese hamster ovary cells show similar kinetics of induction for a number of heat shock proteins (Subjeck et al., 1982). The kinetics of recovery from heat shock are different for hsp108 than for the other chicken hsp's, which can be seen to be switched off more quickly after heat shock (Figure 4A). The reduction in hsp108 RNA concentration during later times of heat shock is also unusual, suggesting that heat shock causes a "once-and-for-all" boost of hsp108 synthesis of a defined size, after which demand for protein is reduced to normal levels.

In addition to the heat shock regulation, hsp108 is also regulated by steroid hormones (M. Baez, unpublished observation). Steroid regulation of heat shock genes also have been observed in hsp22, hsp23, hsp26, and hsp27 of *Drosophila* and hsp85 of HeLa cells (probably equivalent to chicken hsp90; Vitek & Berger, 1984; Ireland et al., 1982; Kasambalides & Lanks, 1983).

Two chicken heat shock proteins (hsp90 and hsp108) have now been found to copurify with progesterone receptor subunits (Catelli et al., 1985; Zarucki-Schulz et al., 1984; Puri et al., 1982). Both are more abundant than the receptor, but there is some evidence that hsp90 exists in cytosol complexed to the receptor (Puri et al., 1982). It is interesting to note that the A subunit of the progesterone receptor is of similar molecular weight to the other novel heat shock protein described in this paper (hsp78). However, preliminary immunologic data suggests that this heat shock protein is also distinct from the receptor (data not shown).

In preliminary experiments we examined whether induction of hsp108 mRNA concentration occurs via an increase in transcription of the gene, using a "runoff" assay of nuclei isolated form cells before or after heat shock. We have been unable to demonstrate pure transcriptional control of the hsp108 heat shock response, so it is possible that control occurs through variation of the rate of RNA turnover as well. It is notable that there are no good homologies to the heat shock promoter consensus elements of Pelham (1982) within 1300 bases upstream of the mRNA cap site (data not shown).

ACKNOWLEDGMENTS

We thank Serlina Robinson, Craig Wilkinson, and Tammy Wynette for excellent technical assistance, Dr. Robert Schwartz for his gift of the plasmid pGAD-28, Cheryl McCarthy for help and forbearance in typing the manuscript, and many colleagues for advice and discussion.

Registry No. DES, 56-53-1.

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Association of Protein C23 with Rapidly Labeled Nucleolar RNA†

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Received March 4, 1986; Revised Manuscript Received May 6, 1986

ABSTRACT: The association of nucleolar phosphoprotein C23 with preribosomal ribonucleoprotein (RNP) particles was examined in Novikoff hepatoma nucleoli. RNA was labeled with [^{3}H]uridine for various times in cell suspensions, and RNP particles were extracted from isolated nucleoli and fractionated by sucrose gradient ultracentrifugation. The majority of protein C23 cosedimented with fractions containing rapidly labeled RNA (RL fraction). To determine whether there was a direct association of RNA with protein C23, the RL fraction was exposed to ultraviolet (UV) light (254 nm) for short periods of time. After 2 min of exposure there was a 50% decrease in C23 as measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses, with no significant further decrease at longer times. When UV-treated fractions were subjected to phenol/chloroform extractions, as much as 30% of the labeled RNA was found in the phenol (protein) layer, indicating that RNA became cross-linked to protein. Similarly, there was an increase in protein C23 extracted into the water layer after irradiation. By SDS–PAGE analyses the cross-linked species migrated more slowly than protein C23, appearing as a smear detected either by [^{3}H]uridine radioactivity or by anti-C23 antibody. With anti-C23 antibodies, up to 25% of the labeled RNA was precipitated from the RL fraction. Dot-blot hybridizations, using cloned rDNA fragments as probes, indicated that the RNA in the RL fraction and the immunoprecipitated RNA contained sequences from 18S and 28S ribosomal RNA. These studies, along with other published work that shows the presence of C23 in fibrillar regions, suggest that protein C23 is associated with nascent preribosomal RNA and that it may be involved in the early stages of preribosomal RNP particle formation.

The nucleolus is the subnuclear organelle where preribosomal RNA is synthesized and ribosome assembly begins (Busch & Smetana, 1970). Ultrastructural studies have shown well-defined regions of the nucleolus, many of which can be related to aspects of cell physiology. For example, fibrillar centers appear as areas of relatively low electron density in transmission electron microscopy and are surrounded by a dense fibrillar component. The fibrillar centers are the apparent location of the genes for preribosomal DNA (Arroua et al., 1982) and RNA polymerase I (Scheer & Rose, 1984; Jordan, 1984). The surrounding dense fibrillar components contain newly synthesized preribosomal RNA with proteins attached (Goessens & Lepoint, 1979; Mirre & Stahl, 1981). The granular components, which are usually distributed throughout the nucleolus, contain the 15-nm preribosomal particles (Jordan, 1984). These ribonucleoprotein (RNP) components consist of preribosomal RNA at various stages of processing associated with unique sets of ribosomal and nonribosomal proteins.

A major nucleolar protein, C23 (also called nucleolin or the 100-kDa nucleolar protein), has a number of unique properties that suggest it may participate in the organization of these nucleolar subcomponents: (a) it is predominantly localized to the nucleolus in interphase cells (Olson et al., 1981; Michalik et al., 1981, (b) it binds silver under conditions in which the nucleolus organizer regions of chromosomes specifically stain with silver (Lischwe et al., 1979; Hubbel et al., 1979), (c) it is found at the nucleolus organizer regions of chromosomes (Lischwe et al., 1981), (d) it is a nonribosomal constituent of nucleolar preribosomal particles (Prestayko et al., 1974; Olson et al., 1974), (e) it contains several highly acidic regions (Mamrack et al., 1977, 1979; Rao et al., 1982), and (f) it contains multiple phosphoryl groups (Mamrack et al., 1977, 1979; Rao et al., 1982).

Of the various nucleolar components, the dense fibrillar regions contain the highest concentration of protein C23 as measured by immunoelectron microscopic methods (Escande et al., 1985; Lischwe et al., 1981; Spector et al., 1984). Like protein C23, the dense fibrillar regions have a high affinity for silver (Hernandez-Verdun et al., 1980; Goessens & Lepoint, 1982) or bismuth salts (Gas et al., 1984), suggesting that these or the enclosed fibrillar centers are interphase

† This work was supported in part by Grant GM28349 (to M.O.J.O.) from the National Institutes of Health.
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0006-2960/86/0425-6258$01.50/0