Expression of a Set of Fish Genes Following Heat or Metal Ion Exposure*

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Elevation of the incubation temperature of Chinook salmon embryo cells from 20 to 24 °C or exposure to heavy metals such as CdCl₂ (5 μM) or ZnCl₂ (100 to 500 μM) induces the reversible expression of a set of heat shock or stress proteins. Continuous exposure of the cells to either metal ions or heat shock results in recovery of protein synthesis to a control-like pattern. Treatment of these cells with either ZnCl₂ or CdCl₂ also induces the protein metallothionein. Heat shock, however, does not induce metallothionein, suggesting that it does not belong to the common group of heat shock or stress proteins. The induction of these stress proteins can be inhibited by pretreatment with actinomycin D, suggesting that their expression is regulated at the transcriptional level. The major stress proteins are detectable in the products of an in vitro translation system programmed with RNA isolated from heat shock- or metal ion-treated cells. A recombinant DNA probe complementary to Drosophila mRNA coding for the 70,000-dalton heat shock protein was found to hybridize to RNA isolated from heat shock- or metal ion-treated cells but not from control cells. The fish mRNA coding for the heat shock protein with a molecular weight of 70,000 appears to be of similar size to the corresponding Drosophila mRNA.

Exposure of prokaryotic and eukaryotic cells to heat shock or other environmental stresses such as arsenite or amino acid analogues (i.e. canavanine) results in the production of a small number of new heat- or stress-induced proteins (1-4). Control of this induction phenomenon in Drosophila has been shown to occur at several levels of gene expression, including the selective synthesis of specific mRNAs (2), and at the level of protein synthesis (5-7). The appearance of the heat shock or stress proteins following thermal stress has been reported in a wide range of organisms, including bacteria (4), protozoa (8), plants (9), insects (2, 10), and a number of eukaryotic cell lines (3, 11, 12), and in rat tissue slices (13). Recently, heat shock proteins have been found in mammalian brain following the induction of hyperthermia in vivo (14). The apparent ubiquitous nature of this temperature stress phenomenon has raised the question of whether these stress proteins have a possible homeostatic function. It has been reported that the induction of stress proteins in a number of organisms such as yeast (15), Dictyostelium (16), and Chinese hamster fibroblasts (17) is correlated with a dramatic increase in the capacity for thermotolerance.

The ability to induce the expression of a set of genes by heat shock or other environmental stresses in a rapid as well as reversible manner provides a useful means by which one can study the mechanisms controlling gene expression in embryonic systems. In this report, we have characterized the stress proteins induced in a fish embryonic cell line by heat shock or metal ion exposure.

MATERIALS AND METHODS

Cell Culture—The Chinook salmon embryo cell line (CHSE-214) was obtained from Dr. R. MacDonald (University of Calgary, Calgary, Alberta). The fish embryo cells were grown in Eagle's minimum essential medium with 5% fetal bovine serum plus 0.15% sodium bicarbonate at 20 °C under an atmosphere of carbon dioxide which maintained the pH of the medium at approximately 7.4 to 7.6. Cell lines were subcultured every 14 days.

Labeling Experiments—Confluent monolayers of cells (1 to 2 × 10⁶) were incubated at 20, 24, or 26 °C or exposed to either CdCl₂ (1 to 10 μM) or ZnCl₂ (100 to 500 μM) at 20 °C for various periods of time. Newly synthesized proteins were labeled by the addition of L-[³⁵S]methionine (Amersham Corp., 1300 Ci/mmol) to a final concentration of 0.2 Ci/ml for the last hour of incubation unless otherwise stated. The fish embryo cells were then scraped from the bottom of the tissue culture dishes and collected by centrifugation. The samples destined for electrophoresis were resuspended in 40 ml of 4% urea, 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, 10% glycerol and briefly sonicated (1 to 2 s). In order to assay for acid-insoluble radioactivity, duplicate 2-ml aliquots were diluted in 150 ml of distilled water containing 50 μg of bovine serum albumin carrier and precipitated on ice with 150 μl of 25% trichloroacetic acid containing 1 mg/ml of methionine. The precipitates were collected on glass fiber filters (Whatman GF/C), washed with 8% trichloroacetic acid containing 1 mg/ml of methionine, and dried. Filters were then counted in a toluene-based scintillation mixture.

In experiments examining the synthesis of metallothionein, cells were labeled with [³²P]orthophosphate (40 μCi/ml of medium; specific activity, 878 Ci/mmol; Amersham Corp.). Samples (10 μl) were carboxymethylated by incubating with 40 μl of 100 mm iodoacetate in 0.5 M Tris-HCl (pH 9.0) for 2 h at 37 °C and then precipitated with acetone. The pellets were then resuspended in sample buffer as described above and assayed for acid-precipitable radioactivity.

RNA Isolation and In Vitro Translation—Fish embryo cell pellets or frozen Drosophila melanogaster (Oregon-R) third instar larvae were homogenized in 7 M urea, 2% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.5) 0.135 M NaCl, and 1 mM EDTA and then extracted twice with an equal volume of chloroform:phenol (1:1). The aqueous phase was extracted with chloroform:methanol (2:1) until a clear interphase was obtained. Total nucleic acid was precipitated with 2.5 volumes of absolute ethanol. Lyophilized aliquots of nucleic acid (5 to
control of expression of the stress proteins is regulated at the transcriptional level.

**Metal Ion-induced Synthesis of Stress Proteins and Metallothionein**—Since a number of other environmental stresses have been shown to induce heat shock or stress proteins in other systems, we examined whether exposure to heavy metals such as Zn²⁺ or Cd²⁺ can induce a set of stress proteins in the fish cell line similar to what is found with heat

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**FIG. 1. Induction of stress proteins in Chinook salmon embryo cells by heat shock:** effect of actinomycin D. Fish cells (1 to 2 × 10⁶ cells) were incubated at either 20 (lanes 1 and 4), 24 (lanes 2 and 5) or 26 °C (lanes 3 and 6) for 2 h. Some samples (lanes 4 to 6) were preincubated with actinomycin D (20 µg/ml) for 30 min prior to heat shock. [³⁵S]Methionine (20 µCi/ml) was added to the culture media for the last hour to label newly synthesized proteins. Cells were collected and then lysed by brief sonication as outlined under "Materials and Methods." Labeled proteins were resolved on 7 to 17% sodium dodecyl sulfate-polyacrylamide gradient slab gels and detected by fluorography. Equivalent amounts of acid-insoluble radioactivity (40,000 cpm) were loaded for each sample. Molecular mass of polypeptides identified by arrows was determined according to electrophoretic migration of standards and is expressed in daltons × 10³.

**FIG. 2. Transient induction of stress proteins during heat shock.** Fish embryo cells were incubated at 24 °C for periods of time ranging from 2 to 24 h. In these samples, [³⁵S]methionine (20 µCi/ml) was added for the last hour. Cell lysis, electrophoresis, and fluorography procedures are as described in the legend to Fig. 1. Lane 1, control (2 h at 20 °C); lane 2, 2 h at 24 °C; lane 3, 5 h at 24 °C; lane 4, 24 h at 24 °C.
shock. Treatment of the fish cells with 5 μM CdCl₂ (Fig. 3) caused an increase in the relative synthesis of a number of proteins ($M_r = 84$, 70, 68, 51, 46, and $28 \times 10^3$, respectively). The induction of these stress proteins by Cd²⁺ is also a transient phenomenon. An enhanced synthesis of the 84,000-dalton protein was detectable within 30 min, maximal at 3 h, and approached control levels within 4 h. The other stress proteins ($M_r = 70$, 68, 51, 46, and $28 \times 10^3$, respectively) were maximal at 30 min to 1 h and approached control levels within 3 to 4 h. A similar induction of stress proteins was also noted with ZnCl₂ (Fig. 4). Increasing the concentration of ZnCl₂ from 100 to 500 μM for 1 h at 20 °C resulted in an increased synthesis of the stress proteins. A similar dose response effect was noted for CdCl₂ concentrations ranging from 1 to 10 μM. Pretreatment of these salmon embryo cells with actinomycin D (20 μg/ml) for 30 min before either ZnCl₂ or CdCl₂ exposure inhibited the induction of these stress proteins (data not shown). Therefore, the expression of the metal ion-induced stress proteins also appeared to be regulated mainly at the transcriptional level.

A comparison of the molecular weights of these stress proteins induced by either heat shock or metal ion exposure revealed that three of these stress proteins ($M_r = 84$, 70, and 68 $\times 10^3$, respectively) are synthesized to similar extents in response to the different stresses. The remaining proteins appear to be preferentially synthesized in response to either heat shock ($M_r = 65,000$ and 41,000) or metal ion exposure ($M_r = 51,000$, 46,000 and 28,000).

The expression of eukaryotic cells to metal ions or glucocorticoid hormones has also been shown to induce a class of metal-binding proteins called metallothioneins (22). Since metallothioneins may have a homeostatic role during metal toxicity or other stresses, it was of interest to determine whether the synthesis of metallothioneins was enhanced following heat shock treatment. Chinook salmon embryo proteins were labeled with [³⁵S]cysteine following exposure to either 5 μM CdCl₂ or heat shock (24 °C), carboxymethylated, and electrophoresed as shown in Fig. 5. An increase in the relative synthesis of a 10,000-dalton protein following CdCl₂ exposure was noted in addition to the other stress proteins mentioned above. This low molecular weight, Cd²⁺-induced protein(s) has been identified as metallothionein by a variety of criteria, such as the ability to bind ¹¹⁶Cd, stability to heat denaturation, characteristic flow properties on a Sephadex G-75 column, and a high $A_{250}/A_{280}$ ratio (details will be published elsewhere). Metallothionein is not detectable until after 24 h of metal ion exposure, while most of the stress proteins are detectable within 30 min. It appears, therefore, that these proteins are not coordinately expressed. Heat shock treatment at 24 °C for periods of time ranging from 2 h to 3 days does not induce metallothionein. Thus, metallothionein does not appear to belong to this common group of stress proteins induced by both heat or metal ion stress.

**In Vitro Translation Studies**—The possibility that these stress proteins are regulated at the transcriptional level is supported by the in vitro translation data presented in Fig. 6. Examination of the spectrum of proteins in the translation...
products of reactions programmed with total RNA from either heat shock-, Cd\(^{2+}\)-, or Zn\(^{2+}\)-treated cells revealed a marked increase in the relative labeling of the 70,000- and 84,000-dalton stress proteins. The increased labeling of the 84,000- and 70,000-dalton proteins reflects an increase in the abundance of their respective mRNAs. These data also suggest that these stress proteins are regulated predominantly at the transcriptional level. Other heat shock- or metal ion-induced stress proteins were not readily detectable in the translation products of the reticulocyte lysate cell-free system. This may indicate that the mRNAs coding for those stress proteins are not translated efficiently in the in vitro system.

RNA-DNA Hybridization Studies—The 70,000-dalton stress protein induced by either heat shock or metal ion treatment is similar in molecular mass to the 70,000-dalton heat shock protein induced in Drosophila. The availability of a cloned genomic DNA fragment from Drosophila comprising the 5'-end of the gene coding for the 70,000-dalton heat shock protein (hsp 70) allowed us to determine if a similar mRNA sequence was induced in fish cells. Fig. 7 shows the translational products of a reticulocyte lysate cell-free translation assay programmed with RNA isolated from either control or heat shocked Drosophila third instar larvae. Clearly, the mRNAs from the major heat shock proteins were induced. Total nucleic acid was then extracted from control, heat shock- or metal ion-treated fish cells as well as from control and heat shocked Drosophila larvae and subjected to electrophoresis on a methyl mercury hydroxide agarose gel. The RNA was transferred to diazobenzyloxymethyl paper and hybridized with the hsp 70 probe labeled by means of nick translation. A typical autoradiogram resulting from this type of experiment is shown in Fig. 8. The cloned hsp 70 probe hybridized extensively to heat shocked Drosophila RNA (lane 2) but not to control RNA (lane 1). The hsp 70 Drosophila probe also hybridized to RNA (Fig. 8, see arrow) isolated from fish cells maintained at either 24 or 26 °C or treated with 500 μM ZnCl\(_2\) but not to RNA isolated from control. A similar result was observed with RNA isolated from CdCl\(_2\)-treated cells (data not shown). The fish mRNA hybridizing to the hsp 70 Drosophila heat shock probe appeared to be of similar size to the corresponding Drosophila mRNA. The trace amount of hybridization of the probe to the higher molecular weight material in all samples represents a small amount of DNA transferred to the diazobenzyloxymethyl paper.

**DISCUSSION**

This study has shown that elevation of the incubation temperature (20 to 24 or 26 °C) of Chinook salmon embryo cells or exposure to metal ions such as Cd\(^{2+}\) or Zn\(^{2+}\) can induce the synthesis of a set of heat shock or stress proteins. A number of these stress proteins (M, \(=\) 84, 70, and 68 x 10^3, respectively) appear to be synthesized to a similar extent after either heat shock or metal ion exposure, whereas others are preferentially synthesized in response to one of the two treatments (i.e. heat shock, M, \(=\) 65,000 and 41,000; metal ion exposure, M, \(=\) 51,000, 46,000, and 28,000). These results suggest that some of the stress proteins, particularly the 70,000-dalton protein, are synthesized to a similar extent in response to both stress factors, whereas the relative synthesis of other stress-induced proteins may be independent upon the type of stress (Figs. 1, 3, and 4).

In a recent report (23) the induction of a similar set of stress proteins in cultured fibroblasts from rainbow trout gonad was observed following heat shock or sodium arsenite exposure. In this latter study, sodium arsenite was found to induce the synthesis of two stress proteins in addition to what was induced with heat shock. Similarly, in chick embryo cells, differences in the molecular weights of proteins whose synthesis was enhanced by arsenite or canavanine have been re-
ported (24). It was also shown that the synthesis of a 35,000-dalton stress protein was greater in cells treated with copper ions than in those given heat shock (24). Furthermore, the stress proteins produced in response to cold shock in Tetrahymena differ from the spectrum of proteins synthesized in response to heat shock (25). It is not known at this time whether this selective stressor-dependent synthesis of specific proteins reflects preferential transcription or control at other levels of gene regulation.

The appearance of the heat shock or stress proteins in fish cells was found to be a transient phenomenon (Figs. 2 and 3). Maintenance of the heat shock conditions (i.e. 24 °C or exposure to Cd²⁺ (5 μM) induced a maximal synthesis of the stress proteins, followed by a recovery period. The 70,000-dalton protein, for example, returned to control levels within 24 h of continued heat shock or within 4 h of Cd²⁺ exposure. A decrease in the rate of synthesis of the 70,000-dalton heat shock protein has also been found in Dicyostelium maintained at elevated temperature (16). In trout fibroblasts, a heat shock temperature of 28 °C resulted in a continued synthesis of the stress proteins (23). The observed differences between our results with Chinook salmon embryo cells and those reported with trout fibroblasts (23) may be a function of the higher temperature used with trout. This is suggested by the report that the synthesis of the Drosophila 70,000-dalton heat shock protein declines within 50 min at 33 °C but persists at a high rate when the temperature is maintained at 37 °C (26). In the latter study, the decrease in the synthesis of the 70,000-dalton protein at 33 °C was associated with an inactivation or degradation of the mRNA coding for this protein. In the fish embryonic cell line, it will be interesting to determine whether the transient induction of the stress proteins and recovery following continuous exposure to elevated incubation temperatures or metal ions are regulated at the transcriptional or translational level.

Metallothioneins are small cysteine-rich proteins that can be induced by heavy metals or glucocorticoid hormones (22). Since these proteins are implicated in homeostasis and heavy metal detoxification, it was of interest to determine whether their synthesis was enhanced in the fish cell line following exposure to different kinds of stresses. The exposure of Chinook salmon embryo cells to CdCl₂ was found to increase the relative synthesis of metallothionein (Fig. 5). This finding agrees with earlier work (27) done on a rainbow trout fibroblast cell line. Enhanced metallothionein synthesis, however, was not detected following heat shock. Therefore, metallothionein does not appear to belong to the common group of stress proteins induced by metal ion or heat shock treatment. Furthermore, the metal ion-induced stress proteins and metallothionein are not coordinately expressed by CdCl₂ exposure since the stress proteins are detected within 30 min, whereas enhanced synthesis of metallothionein is not detectable in this cell line until 24 h.

The heat- and metal ion-induced stress proteins appear to be regulated mainly at the transcriptional level. This suggestion is based on the finding that pretreatment of the salmon embryo cells with actinomycin D can prevent the induction of the stress proteins by either heat shock or metal ion exposure (Fig. 1). This is also supported by the in vitro translation experiments which showed an increase in the abundance of mRNAs coding for the 84,000- and 70,000-dalton stress proteins following either heat shock or metal ion exposure (Fig. 5). As will be discussed below, an increase in the abundance of the mRNA coding for the 70,000-dalton stress protein was also detected by molecular hybridization of RNA isolated from heat-shocked or metal ion-treated fish cells to a cloned DNA probe comprising the 5' portion of a Drosophila hsp 70 gene. Transcriptional regulation of stress proteins during heat shock or sodium arsenite exposure has also been suggested for rainbow trout fibroblasts (23).

The predominant stress protein found in the Chinook salmon embryo cells is the 70,000-dalton species. We and others (23) have also found a similar result with a fibroblast cell line derived from rainbow trout gonad following heat shock. A stress protein of similar molecular weight has been reported in Drosophila (2), HeLa cells (28), chicken embryonic fibroblasts (12), sea urchins (29), Dicyostelium (16), rabbit (14), and rat (13). It is tenable that the 70,000-dalton heat shock proteins found in these different organisms are structurally and functionally related. This possibility is supported by the finding that antibodies raised against the 70,000-dalton protein of the chicken embryo fibroblast have been found to cross-react with heat shock extracts from a wide range of organisms including yeast, Drosophila, Xenopus, and mouse (30). The results obtained in this study with salmon embryo cells support this contention since the mRNAs coding for the 70,000-dalton heat shock or stress protein in fish and Drosophila are structurally similar. This is based on the fact that a cloned genomic DNA fragment encoding the 5' end of the Drosophila hsp 70 heat shock gene hybridized very efficiently to the RNA isolated from heat-shocked or metal ion-treated fish cells (Fig. 8). The fish mRNA which hybridized to the Drosophila heat shock probe was approximately the same size as the mRNA coding for the Drosophila heat shock protein.

This study has shown that a set of heat shock or stress genes can be expressed in an embryonic fish cell line by exposure to heavy metals as well as to a moderate increase in the incubation temperature. An interesting observation reported in this study concerns the transient nature of this induction phenomenon during continued environmental stress. The availability of recombinant DNA probes complementary to heat shock and other genes will enable us to determine the fate of cellular mRNAs during this recovery process. This approach will aid in determining whether the mechanism of recovery or accommodation to the environmental stresses resides at the transcriptional or translational level.

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