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Z. Ju · R.A. Dunham · Z. Liu

Differential gene expression in the brain of channel catfish (*Ictalurus punctatus*) in response to cold acclimation

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Abstract Aquatic ectotherms can adapt to a wide range of temperature changes, but the molecular mechanisms that underlie this adaptability are not well understood. We identified genes that are differentially expressed in the catfish (*Ictalurus punctatus*) brain using a cDNA microarray approach to gain an initial understanding of adaptation to low temperature. Among 660 genes analyzed, 61 were differentially expressed when compared at 12°C and 24°C. Gene induction was rapid, occurring within 2 h of the temperature shift. The major categories of differentially expressed genes included (1) genes for chaperones such as Hsp70 and Hsp70/Hsp90 organizing protein; (2) genes for transcription factors and gene products involved in signal transduction pathways such as zinc-finger proteins, calmodulin kinase inhibitor, the nuclear autoantigen SG2NA, interferon regulatory factor 3, and inorganic pyrophosphatase; (3) genes involved in lipid metabolism such as TB2 and acyl CoA binding protein; and (4) genes involved in the translational machinery such as ribosomal proteins. Some genes were induced transiently, whereas others were induced in an enduring fashion. Several genes, primarily ribosomal protein genes, were down regulated, indicating reduced metabolic activities after extended incubation at the low temperature. Thus channel catfish respond to low temperature by adjusting expression of a large number of genes. The rapid induction of proteins involved in signal transductions and chaperones suggests that both de novo synthesis of cold-induced proteins and modification of existing proteins are required for adaptation and tolerance of catfish to low environmental temperature.

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Z. Ju · R.A. Dunham · Z. Liu (✉)
Fish Molecular Genetics and Biotechnology Laboratory,
Department of Fisheries and Allied Aquacultures
and Program of Cell and Molecular Biosciences,
203 Swingle Hall, Auburn University,
Auburn, AL 36849, USA
E-mail: zliu@acesag.auburn.edu
Tel.: +1-334-8444054
Fax: +1-334-8449208

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Introduction

Temperature is a major seasonal environmental factor that can also undergo daily fluctuations and short erratic lows and highs. These types of temperature stresses are associated with disease and mortality in catfish. Aquatic ectotherms exist in environments with temperatures ranging from –2°C in the polar oceans to over 40°C in hot springs. A single fish species may even adapt seasonally to temperatures from near freezing to over 36°C in temperate freshwater systems. They must be able to sense transient fluctuations as well as seasonal changes in temperature and to respond to these changes by actively adjusting their physiology to fit the ambient temperature regime (Browse and Xin 2001). Low temperature in late fall or early winter is the main trigger for cold acclimation in plants (Thomashow 1999), but little is known about cold acclimation in fish. The great adaptability of fish to a wide range of temperatures is truly amazing in comparison to the span of a few degrees that a homeotherm's tissue can survive (Dunn 1987).

Catfish is the major aquaculture species in the United States. It is a temperate species, and must cope with seasonal temperature changes from near freezing during winter in the North to over 36°C in the summer in the South. Even in the Southern United States, winter water temperature routinely reaches only 10–12°C, and can decrease to near 0°C. Variation in temperature may greatly affect the metabolic rates in cells. As a result, catfish exhibit reduced feeding and growth during the winter months.

Changes in gene expression under acclimation to cold have been extensively studied in teleost fish. Many genes are up-regulated in response to cold acclimation, including those for cytochrome *c* oxidase (Hardewig et al. 1999), cytochrome P450 (Kloepper-Sams and Stegeman 1992), antifreeze protein (Pickett et al. 1983), metallothionein-1

(Beattie et al. 1996), carnitine palmitoyltransferase I (Rodnick and Sidell 1994), adenine nucleotide translocase (Roussel et al. 2000), and Wap65 (a homolog of the mammalian homopexins, Kikuchi et al. 1995). However, a systematic analysis of changes in gene expression in response to temperature shifts has not yet been carried out, partly because of the technical difficulties.

Our ability to detect differentially expressed genes has greatly improved recently. Earlier research used the techniques of representational difference analysis (Welford et al. 1998), and differential display (Liang et al. 1992). Recent development of cDNA microarrays and oligonucleotide arrays has facilitated the discovery of differentially expressed genes under various conditions (Schena et al. 1995; Shalon et al. 1996; Yue et al. 2001). Such technologies allow the identification of differentially expressed genes in tissues and during development (Bryant et al. 1999; Zirlinger et al. 2001), after infection or wounding (Ichikawa et al. 2000; Reymond et al. 2000), or under pathological conditions (Welford et al. 1998; Hakak et al. 2001). The major advantage of microarray technology is that it allows the simultaneous examination of large numbers of genes. However, such arrays are most suited for model species for which cDNA microarrays are available or where the oligonucleotide microarrays are relatively less expensive because the number of potential users is large. For aquaculture species such as catfish, large-scale, high-density microarrays are not yet available. Identification of differentially expressed genes during cold acclimation using low-density cDNA microarrays as a means of systematic analysis of gene expression is reported here. Among 660 catfish brain genes analyzed, 61 were found to be differentially expressed.

Materials and methods

Animals

Experimental channel catfish (*Ictalurus punctatus*) were produced in early June and grown in a 0.04-ha earthen pond until experiments were initiated in mid-October. The fish were randomly divided into two groups. One group was kept in troughs in the hatchery and held at a constant temperature of 24°C. The other fraction remained in the pond. Water temperature in the pond varied with ambient temperature from mid October to early December. Fish typically acclimated to cold during this period in Alabama. Water temperature in the pond was 12 ± 2°C when the fish were sampled. Brain tissues were collected from 5–10 fish from both groups to compare gene expression patterns between the fish held at a constant temperature of 24°C and under natural cold conditions.

Temperature treatment

After growth at 24°C in the hatchery for 6 weeks, the 6-month-old fish were subjected to decreased temperature. The water in the troughs located in the hatchery was gradually mixed with pond water (12°C) to reduce the water temperature at a rate of 4°C per hour, in order to avoid temperature shock and mortality. After 3 h, the temperature reached 12°C. Brain tissues were collected from fish kept at 24°C, and from fish kept at 12°C for 0, 2, 24 and 48 h. The brain tissues were stored in liquid nitrogen before use.

mRNA extraction

Brain tissues were ground with a mortar and pestle, and then homogenized in RNA extraction buffer with a hand-held tissue tearor (Model 985-370, Biospec Products) following the guanidium thiocyanate method (Chomczynski and Sacchi 1987). mRNA was purified from total cellular RNA using the Poly (A)+ Pure kit (Ambion, Cat. No. 1918) according to the manufacturer's instructions, except that two rounds of purification were performed.

Preparation of cDNA microarray

Expressed sequence tags (ESTs) used for preparation of the low-density microarray were previously reported (Ju et al. 2000). The ESTs were arrayed onto nylon membranes using a 384 multi-blot replicator (V and P Scientific). Every EST was spotted twice in adjacent positions onto the same membrane to provide duplicate EST arrays. The cDNA on the arrayed membranes was then denatured in 0.5 M NaOH for 3 min, washed by floating the membrane on 0.5 M TRIS-HCl (pH 7.5) and then 0.5 M TRIS-HCl/1.5 M NaCl (pH 7.5) for 3 min, respectively. The DNA was cross-linked to the membrane by using the Auto Cross Link function of the UV StratatlinkerTM 2400 (Stratagene).

Probes, labeling, and hybridization

mRNAs were isolated from brain tissues of fish subjected to the following treatments: 12°C 0 h, 12°C 2 h, 12°C 24 h, 12°C 48 h, 24°C and 12°C. Double-stranded cDNA was made from the mRNA using the reagents provided in the pSPORT-1 SuperScript plasmid cloning system (Gibco-BRL) following the manufacturer's instructions except that an oligo $d(T)_{18}$ was used as primer. Aliquots (1 μg) of cDNA were used for labeling. Mixed probes were obtained by labeling the synthesized cDNAs using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals) following the manufacturer's instructions, except that 100 nM oligo $d(T)_{18}$ was used in the prehybridization and hybridization reactions to block poly$(A)^+$ sequences within the cDNA arrayed on the membrane. All hybridizations were repeated at least twice (a total of at least four spots per gene).

Data acquisition and statistical analysis

Hybridized membranes were exposed to Kodak film for 3–5 min. The developed film was scanned using a MagicScan version 4 scanner (UMAX Technologies), and then analyzed using Kodak 1D Image Analysis Software (Eastman Kodak). Background was subtracted from the signal intensities. Three genes were used as internal controls: creatine kinase, immunoglobulin heavy chain, and Sec61 (transport protein) gamma. Variation in hybridization intensities among membranes was calibrated using the three internal controls. Since constant hybridization signals were obtained with the three internal control genes, they were used to obtain relative expression levels under each temperature treatment. Relative expression levels were expressed as the ratio of the signal intensity for the clone of interest to the average intensity for the three control genes. Standard deviations were calculated from the four replicates and Student's t-test was used to determine statistical significance using JMPIN software (SAS Scientific).

Results

Development of low-density microarrays

To study genes that are differentially expressed in the brain using micoarrays, 1201 expressed sequence tags

(ESTs) were previously sequenced from the brain of channel catfish (Ju et al. 2000). That work led to identification of 660 unique genes from the channel catfish brain. In absence of a microarray spotter, our first task was to develop low-density microarrays. This was accomplished by first transferring all cDNA clones to 384-well plates. The cDNAs were then spotted onto nylon membranes using a multiple blot replicator (manual arrayer). With a pin guide of two positions in each dimension, the manual arrayer allowed spotting of cDNAs at a density of 1536 (4×384). This capacity allowed placement of repeated double spots for all 660 genes on each membrane (Fig. 1).

Identification of five types of differentially expressed genes in catfish held at different temperatures

The genes expressed in channel catfish held at 12°C in the pond were compared to those that were active in fish held at 24°C, in order to understand the molecular basis of cold adaptation. Two sets of experiments were conducted. First, patterns of gene expression were compared between channel catfish that had been held at ~12°C and 24°C for 6 weeks. Any differences between these two groups of fish is likely to represent genes that are differentially expressed in a more enduring way. Second, channel catfish that had been held at 24°C for 6 weeks were shifted to a temperature of 12°C. Gene expression was then analyzed at various times after the shift to determine the temporal pattern of gene induction. Among the 660 genes assayed, a total of 61 were differentially expressed (Table 1).

Five categories of differentially expressed genes were observed (Fig. 2): (1) Genes that were transiently induced when catfish were exposed to low temperature of 12°C; these genes were expressed at low levels at 24°C. As soon as the channel catfish were acclimated to the low temperature, these genes were highly induced, but after a short period of time, the expression returned to low levels comparable to those before induction. (2) Genes that were durably induced when channel catfish were exposed to a temperature of 12°C; these genes were very similar to the genes in the first category, but the induction lasted for an extended period, as enhanced expression was observed even after 6 weeks of incubation at the low temperature. (3) Genes that were transiently up-regulated when channel catfish were exposed to the cold temperature; these genes were expressed at reasonably high levels at 24°C. As soon as the channel catfish were acclimated to low temperature, the levels of expression of these genes increased further, but soon returned to levels comparable to those before induction. (4) Genes that were durably up-regulated; these genes were expressed at a reasonably high levels at 24°C. After catfish were exposed to the low temperature, they were expressed at even higher levels; and the induced high-level gene expression lasted for an extended period as analyzed at the end of six weeks. (5) Genes that were down-regulated after exposure to the low temperature (Fig. 2).

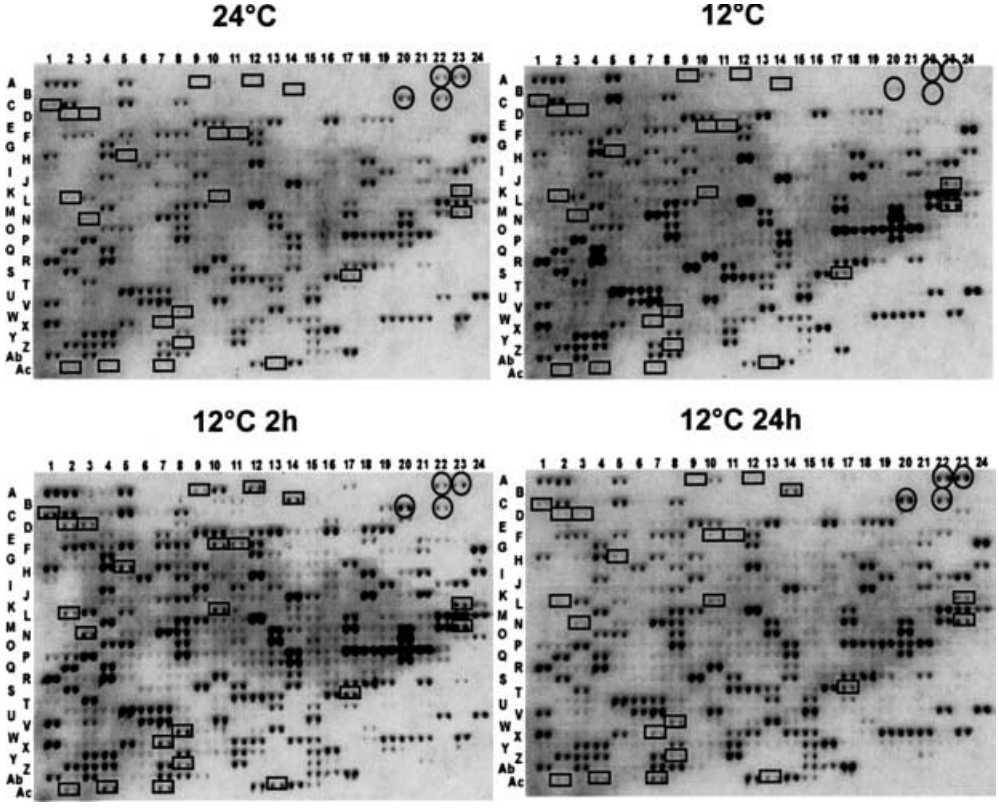

Fig. 1. Low-density cDNA microarray hybridization. Four microarray filters are shown: 24°C, hybridized to probes from fish incubated at 24°C for 6 weeks; 12°C, hybridized to probes from fish incubated under natural conditions in a pond for 6 weeks at a temperature of 12°C; 12°C 2 h, hybridized to probes isolated from fish 2 h after a shift from 24°C to 12°C; and 12°C 24 h, hybridized to probes obtained from fish 24 h after a shift from 24°C to 12°C. The *rectangles* indicate genes that were up-regulated genes on transfer to the low temperature of 12°C; *circles* indicate genes that were down-regulated genes at 12°C. For details of probe preparation, see Materials and methods

Table 1. Summary of the microarray-based analysis of gene expression of catfish under different incubation temperatures

Class of genes	Number
Total number of genes analyzed	660
Known genes	330
Unknown genes	330
Transiently induced genes	5
Durably induced genes	23
Transiently up-regulated genes	11
Durably up-regulated genes	14
Down-regulated genes	8
Total number of differentially expressed genes	61

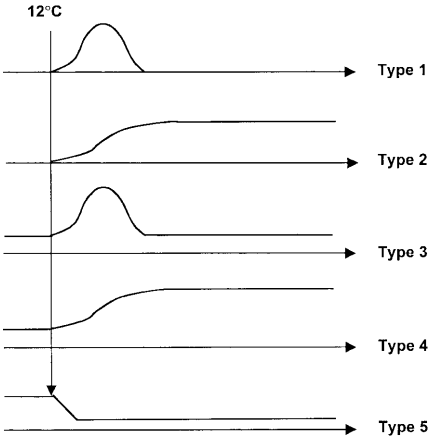

Fig. 2. Types of differentially expressed genes. Type 1, transiently induced genes that were expressed at low levels before the temperature shift; Type 2, durably induced genes that were expressed at low levels before the temperature shift; Type 3, transiently induced genes that were expressed at reasonably high levels before the temperature shift; Type 4, durably induced genes that were expressed at reasonably high levels before the temperature shift; and Type 5, genes that were down-regulated at the low temperature of 12°C

Transiently induced gene expression

Six genes were transiently induced after exposure to cold temperature of 12°C (Type 1, Fig. 2). Only one of the five genes belonging to this category was a known gene (Table 2). The heat shock protein 70 (Hsp70) was rapidly induced to a high level after the temperature shift (Fig. 3). It was already fully induced during the temperature shift period even before the water temperature reached 12°C, but high-level expression lasted for less than 2 h and returned to its normal level of expression after 2 h at 12°C. A similar induction pattern was observed for the other four, unknown, genes (Ju et al. 2000). Of the four unknown genes, IpBrn00186 and IpBrn02073 exhibited a very similar pattern of expression to Hsp70, while the induction of IpBrn00178 and IpBrn00203 was slower (Fig. 3).

Durably induced genes

Large numbers of genes were induced in a durable manner after exposure to low temperature (Type 2, Fig. 2), including four known genes and 19 unknown genes (Table 3). The four known genes are predicted to encode a chaperone, a transcriptional factor, and proteins involved in signal transduction. The stress-inducible phosphoprotein I, also known as the Hsp70/Hsp90 organizing protein, was highly induced. IpBrn00501 was a highly induced gene that shared significant similarity with the human hypothetical zinc-finger protein KIAA0296, a hypothetical transcriptional factor. The other two genes were phosphatidic acid phosphatase type 2c and calmodulin kinase II inhibitor protein, both of which are involved in signal transduction pathways. While a large number of genes were differentially expressed at different incubation temperatures, most of these genes cannot yet be identified by BLAST homology comparisons.

Table 2. Genes that were transiently induced genes in the brain of channel catfish when following a temperature shift from 24°C to 12°C

Clone No.	Accession No.	Predicted gene product
IpBrn00166	BE212609	Heat shock protein 70
IpBrn00178	BE212510	Unknown
IpBrn00203	BE212470	Unknown
IpBrn00186	BE212515	Unknown
IpBrn02073	BE213205	Unknown

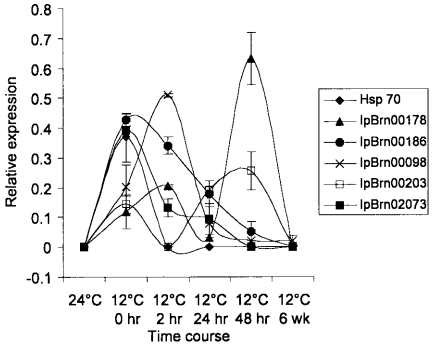

Fig. 3. Expression patterns of the six genes that were transiently induced at the low temperature of 12°C

Transiently up-regulated genes

Eleven genes were found to be transiently up-regulated after exposure to the low temperature (Type 3, Fig. 2). These genes included seven known genes and four unknown genes (Table 4). The known genes included two ribosomal protein genes (L35 and L27), and genes predicted to encode an acyl CoA-binding protein, an inorganic pyrophosphatase, nuclear autoantigen GS2NA, beta-actin, and a protein that shared high identity with the hypothetical human zinc-finger protein KIAA0479. The time course of induction and levels of induction of these 11 genes are shown in Table 4. Particularly worth mentioning is the extent of induction for

Table 3. Genes that are differentially expressed in the brain of channel catfish incubated at 12°C compared to 24°C

Clone No.	Accession No.	Predicted gene product
IpBrn02128	BE213181	Stress-induced-phosphoprotein 1 (hsp70/hsp90 organizing protein)
IpBrn00092	BE212491	Phosphatidic acid phosphatase type 2c
IpBrn00501	BE212631	Hypothetical zinc-finger protein KIAA0296
IpBrn00824	BE212790	Calmodulin kinase II inhibitor protein
IpBrn00173	BE212622	Unknown
IpBrn00350	BE212540	Unknown
IpBrn00368	BE212719	Unknown
IpBrn00634	BE212734	Unknown
IpBrn00646	BE212732	Unknown
IpBrn00887	BE212819	Unknown
IpBrn02102	BE213215	Unknown
IpBrn02091	BE213213	Unknown
IpBrn01602	BE213137	Unknown
IpBrn02111	BE213220	Unknown
IpBrn01682	BE213158	Unknown
IpBrn02124	BE213226	Unknown
IpBrn01430	BE213085	Unknown
IpBrn00098	BE212493	Unknown
IpBrn01173	BE212880	Unknown
IpBrn00102	BE212726	Unknown
IpBrn00876	BE212815	Unknown
IpBrn00879	BE212816	Unknown
IpBrn01056	BE212834	Unknown

the KIAA0479 ortholog and the unknown gene IpBrn01062, both of which were induced over 10-fold at some time points. The common feature of this category is that all of these genes were induced rapidly within the first two hours after the temperature shift, but these high levels of induced expression did not last for extended periods.

Durably up-regulated genes

Fourteen genes were found to be up-regulated in a durable manner (relative to fish incubated at 24°C) after a shift to 12°C followed by continuous culture under the cold conditions for 6 weeks. Three of the 14 genes were known genes and 11 were unknown genes. The known genes encode interferon regulatory factor 3, a TB2 homolog, and the 12S mitochondrial rRNA. Most of these genes were highly induced (Table 5). In particular, the unknown gene IpBrn00210, was dramatically induced. Depending on the time point considered, IpBrn00210 was induced 12–36-fold, with the highest induction being observed 1 h after the shift to 12°C. Induction of the mitochondrial 12S rRNA was not nearly as dramatic, but enhanced expression was consistently observed (Table 5).

The level of durable up-regulation at 12°C varied from two-fold to almost 18-fold (Table 5). Almost all of the differentially expressed genes were already induced during the process of temperature shift from 24°C to 12°C, indicating that gene induction after exposure to reduced temperature was rapid. Usually, the highest level of induction was reached after 1 h at 12°C (Fig. 4). When levels of gene induction were averaged over all 14 up-regulated genes, the highest level of gene induction occurred during the period from 1 h to 1 day at 12°C (Fig. 4).

Down-regulated genes

Several genes were down-regulated at 12°C as compared to the expression level at 24°C (Table 6). Most of these genes encode products involved in the translational machinery, including the ribosomal proteins S13, S14, S15A, L30, L31, and the 16S mitochondrial rRNA. One

Table 4. Timecourse of induction of genes that are transiently up-regulated in the brain of channel catfish following transfer from 24°C to 12°C

Clone No.	Predicted product	Temperature regime[a]				
		12°C 0 h	12°C 2 h	12°C 24 h	12°C 48 h	12°C 6 wk
IpBrn00337	Ribosomal protein L35	0.4	3.0	1.3	2.4	1.6
IpBrn00089	Ribosomal protein L27	2.4	1.6	3.2	3.3	1.7
IpBrn02079	Acyl-CoA-binding protein	1.0	3.5	4.1	5.3	0.8
IpBrn02087	Human KIAA 0479 protein	6.1	6.4	7.8	12.7	0.3
IpBrn00218	Beta actin	3.5	3.0	2.8	3.2	0.7
IpBrn00059	Inorganic pyrophosphatase	0.0	3.3	2.7	1.1	0.7
IpBrn00865	Nuclear autoantigen GS2NA	1.6	3.9	2.7	1.9	1.8
IpBrn01062	Unknown	15.9	5.9	6.4	7.2	1.4
IpBrn01483	Unknown	0.9	4.2	1.6	2.8	1.5
IpBrn01612	Unknown	3.1	3.6	3.1	4.3	1.5
IpBrn01632	Unknown	2.5	3.6	4.3	3.4	1.4
IpBrn00063	Unknown	3.7	5.6	5.1	6.6	0.055

[a]Numbers the degree of induction at the indicated time points relative to the expression level at 24°C

Table 5. Time course of induction of genes that are durably up-regulated following transfer of channel catfish from 24°C to 12°C

Clone No.	Predicted product	Temperature regime				
		12°C 0 h	12°C 1 h	12°C 24 h	12°C 48 h	12°C Pond
IpBrn01665	Interferon regulatory factor 3	6.1	4.5	5.6	1.8	3.8
IpBrn02045	TB2 gene homologue	7.2	11.2	8.3	6.7	6.0
IpBrn00301	12S mitochondrial rRNA	1.5	4.0	2.8	1.9	2.3
IpBrn00355	Unknown	3.8	6.0	6.1	5.1	4.6
IpBrn01458	Unknown	2.4	3.2	2.8	2.1	2.4
IpBrn00210	Unknown	12.6	36.3	19.8	11.5	17.7
IpBrn00211	Unknown	5.6	9.6	7.4	3.1	4.7
IpBrn00216	Unknown	2.2	5.7	3.2	4.2	3.9
IpBrn01588	Unknown	4.4	5.1	3.8	3.6	3.4
IpBrn01526	Unknown	1.7	3.3	4.0	2.7	2.0
IpBrn01532	Unknown	3.3	3.1	4.1	4.8	2.0
IpBrn01050	Unknown	2.6	3.9	3.5	2.2	3.5
IpBrn01561	Unknown	4.9	3.9	5.2	2.5	4.1

[a]Numbers indicate the degree of induction at the indicated time points relative to the expression level at 24°C

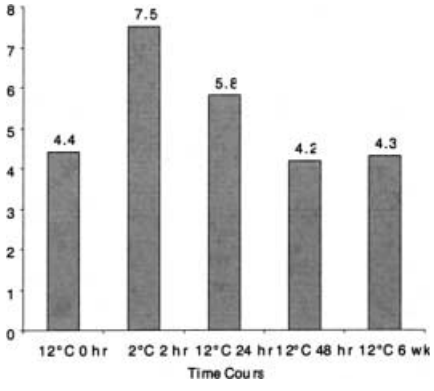

Fig. 4. Summary of the time course of expression of the 14 durably up-regulated genes (Table 5) showing that on average the maximum level of induction occurred 2 h after the shift from 24°C to 12°C

unknown gene, IpBrn00063, was down-regulated at the low temperature. What is interesting is the hypothetical human protein KIAA0479 exhibited a dramatic transient up-regulation as discussed above, but was down-regulated after an extended period of incubation at the low temperature.

Discussion

Aquatic ectotherms have an extraordinary ability to adapt to various water temperatures in different seasons. Much research has been conducted in order to identify differentially expressed genes that enable fish to cope with low temperatures, but a systematic analysis of differentially expressed genes has not been possible. In the present study, the molecular basis for cold temperature acclimation in channel catfish was examined using cDNA microarrays. Despite the relatively small numbers of genes examined, this work is the first to demonstrate that large numbers of genes respond to changes in the aquatic environment in a teleost fish. Among the 660 genes examined, 61 (almost 10%) showed some change in expression level at the two temperatures. Although the relative proportion of genes involved in cold acclimation may decrease as the total number of genes analyzed increases, our results illustrate the dynamic nature of the transcriptome. Clearly, while genomes are relatively stable, the transcriptome is highly variable under different environmental and physiological conditions.

The majority of the genes that were differentially expressed during cold acclimation could not be identified on the basis of homology searches. Only 23 of the 61 differentially expressed genes could be identified by BLAST analysis. Because of the large numbers of unknown genes involved in cold acclimation, it is not yet possible to draw a clear picture of the molecular events that lead to adaptation or tolerance of the organism to adverse environmental conditions. Systematic examination of differentially expressed genes using microarray may provide a very powerful tool with which to address the very complex molecular events that result from a very simple environmental change. We hypothesize that a number of molecular events must occur to prepare the organism for environmental stresses (Fig. 5). These events probably include a cascade of signal transduction, activation of transcriptional factors that direct synthesis of new proteins to cope with the low temperatures, and synthesis of molecular chaperones to modify the existing cellular proteins in response to the temperature shift. Many of these molecular events are rapid and transitory, but expression of some genes may be persistently induced in order to cope with continued low water temperatures. The responses of the identifiable genes in our study support the hypotheses mentioned above.

The most striking group of known genes induced at low temperature are predicted to encode chaperones. Thus, heat shock protein 70 was rapidly and dramatically induced after the temperature shift. However, induction was transient. Two hours after the temperature shift, expression of the Hsp70 gene returned to the

Table 6. Genes that are down-regulated at 12°C (relative to 24°C) in the brain of channel catfish

Clone No.	Accession No.	Predicted product	Degree of down-regulation
IpBrn01619	BE212956	Ribosomal protein S13	16.1
IpBrn01371	BE212957	Ribosomal protein S14	11.9
IpBrn00086	BE212562	Ribosomal protein S15A	32.4
IpBrn00606	BE212554	Ribosomal protein L30	52.1
IpBrn01445	BE212950	Ribosomal protein L31	7.7
IpBrn00066	BE212567	16S mitochondrial rRNA	33.2
IpBrn02087	BE213212	Human KIAA0479 protein	4.5
IpBrn00063	BE212460	Unknown	18.3

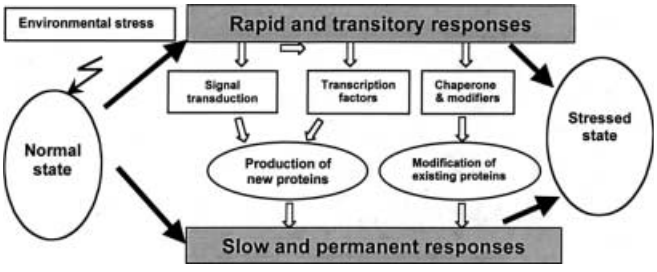

Fig. 5. Schematic representation of the molecular events proposed to lead to adaptation or tolerance to adverse environmental conditions

normal level. Transient elevation of Hsp70 expression was also observed when channel catfish cells in tissue culture were incubated at 37°C (Luft et al. 1996). Another induced chaperone protein was the stress-induced phosphoprotein 1 (STI1), also known as the Hsp70/Hsp90 organizing protein. In murine species, this stress-inducible protein serves as a co-chaperone and functions as a physical link between Hsp70 and Hsp90 by mediating the formation of the mSTI1/Hsp70/Hsp90 chaperone heterocomplex (Longshaw et al. 2000). It is likely that other chaperones in addition to these two are induced by the fall in temperature, but were not identified because of the limited number of genes examined.

The second group of differentially expressed genes included genes that are involved in lipid metabolism. This is expected, since the properties of cellular membranes change with temperature, and organisms must alter the lipid composition of membranes to maintain function. It has been shown that B and T lymphocytes, as well as thrombocytes and erythrocytes, in channel catfish respond to low temperature by increasing the level of unsaturated fatty acids in their phospholipids (Bly et al. 1986). Thus, genes that were differentially expressed at low temperature included phosphatidic acid phosphatase (PAP) type 2c, acyl-CoA-binding protein (ACBP), and the TB2 gene. PAP converts phosphatidic acid to diacylglycerol, thus regulating the de novo synthesis of glycerolipids and also signal transduction mediated by phospholipase D. The type 2 PAPs appear to metabolize a wide range of lipid mediators derived from both glycero- and sphingolipids (Kanoh et al. 1999). The TB2 gene (Kinzler et al. 1991) is the human homolog of the yeast Yop1p, which negatively regulates cell growth. Disruption of Yop1p has no apparent effect on cell viability, while overexpression results in cell death, accumulation of internal cell membranes, and a block in membrane trafficking (Calero et al. 2001). These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane trafficking (Calero et al. 2001). Overexpression of ACBP results in greater incorporation of palmitic acid predominantly into the triglyceride fraction, suggesting a role for ACBP in the partitioning of fatty acids between esterification reactions leading to the formation of neutral lipids and beta-oxidation (Yang et al. 2001). In yeast, depletion of ACBP dramatically reduces the content of C26:0 species in total fatty acids, and sphingolipid synthesis is reduced by 50–70%. ACBP-depleted cells accumulated 50- to 60-nm vesicles and autophagocyte-like bodies and showed strongly perturbed plasma membrane structures, suggesting that ACBP plays an important role in fatty acid elongation and membrane assembly and organization (Gaigg et al. 2001). Our results strongly support the hypothesis that at low temperatures, fish must activate many genes involved in membrane metabolism in order to maintain membrane fluidity and function.

The third notable group of induced genes included genes involved in the translational machinery. Although variation was observed, in general, up-regulation occurred immediately after the shift to low temperature, and a general down-regulation occurred on extended incubation at the low temperature. The rapidly enhanced expression observed immediately after the temperature shift may be induced by an increased demand for the de novo synthesis of proteins, such as molecular chaperones, required to cope with the environmental change. However, with extended incubation at the low temperature, the organism may adapt to the changed environment, and since overall metabolic rates will fall at the low temperature, much lower levels of protein synthesis will be required following cold acclimation.

The identities of several induced genes suggest that multiple signal transduction pathways are involved in cold acclimation. The nuclear autoantigen SG2NA was transiently induced after temperature shift from 24°C to 12°C, whereas calmodulin kinase II inhibitor protein was durably induced. SG2NA has been reported to be localized in the nucleus and may function as a scaffold protein involved in $Ca2+$-dependent signal transduction pathways (Moreno et al. 2000). Calmodulin kinase II inhibitor protein serves to inhibit the function of the calmodulin-dependent protein kinase II (CaM-KII),

which is a widely distributed protein kinase that is particularly abundant in neuronal tissues (Chang et al. 1998). Binding of $Ca2^{+}$/calmodulin causes a conformational change in CaM-KII which results in its activation (Mukherji et al. 1994). Induction of the calmodulin kinase II inhibitor protein and SG2NA nuclear autoantigen strongly suggests that calcium signal transduction pathways are involved in cold acclimation in fish. In addition, inorganic pyrophosphatase was also transiently induced, which may be a result of a high level of energy metabolism or of high levels of cyclic nucleotide metabolism (Yang and Wensel 1992). Taken together, these data suggest that cyclic AMP (cAMP) and cyclic GMP (cGMP) could also be potentially involved as messengers in the process of cold acclimation in fish. In plants, multiple signal transduction pathways have been suggested to participate in cold acclimation (Browse and Xin 2001).

Expression of the interferon regulatory factor (IRF) 3 gene was significantly enhanced in the low temperature. This may be one important lead in the search for connections between responses to infection and environmental stresses. Cold stress causes immune-suppression in mice (Cheng et al. 1990) and such immunosuppression at low temperature has also been demonstrated in channel catfish (Bly and Clem 1991). Localized and systemic cytokine production in virus-infected cells plays an important role in the outcome of viral infection and pathogenicity. The IRF is a critical mediator of cytokine gene transcription. Recent studies have focused on the IRF-3 gene product as a direct transcriptional regulator of type 1 interferon (IFN-alpha and IFN-beta) and RANTES cytokine activation in response to virus infection (Lin et al. 1999, 2000). The function of IRF in coping with abiotic stresses is not yet known. Further studies of IRF-mediated responses to adverse environment or immunity are needed.

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