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Analysis of Environmental Stresses Using DNA Chips and Its Application in Cell-Based Biosensor Development

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Environmental stresses caused by various toxic chemicals on *Escherichia coli, Saccharomyces cerevisiae* (Yeast), and *Oryzias latipes* (Japanese Medaka fish) have been evaluated using both DNA chip and real time PCR analyses. The transcriptional responses of *E. coli* were studied with the DNA chip and real time PCR after exposure to various toxic chemicals, including mitomycin C, H_2O_2, phenolics, and others. RT-PCR was used as a major analysis tool for evaluating yeast responses to BaP. In addition, phenol and three EDCs, *i.e.*, 17-beta-estradiol, nonylphenol, and bisphenol A, have been used to study the toxicogenomic responses of Medaka fish with both DNA chip and real time PCR analyses. All the studies indicate that both the DNA chip and real time PCR are appropriate tools to study and elucidate a global view of gene expression profiles, even do so quantitatively, in response to environmentally toxic chemicals.

Introduction

All life on earth has responses and/or adaptation mechanisms for when they are exposed to various stressful conditions, such as high temperatures, oxidative radicals, phenolics and other organic chemicals, heavy metals and ionizing radiation. The effects of these environmental stresses on the genetic level have been elucidated or studied using RT-PCR and transcription based reporter expression systems, such as firefly luciferase, beta-galactosidase, and recently bacterial bioluminescence and GFP. However, these previous testing strategies only measure the effects of pollutants exposure on single or multi-gene level. Toxicogenomics, toxicology with genomics, describes the new research area attempting to define how the regulation and expression of genes mediate the toxicological effects associated with exposure to environmental pollutants [Steiner and Anderson, 2000]. With the introduction of most updated high-throughput and quantitative analysis tools, such as real-time PCR and DNA chips, the tools for studying toxicogenomics are widely available and allows one to elucidate a global view of gene expression profiles, even quantitatively, in a given cell or tissue type after exposure to environmental pollutants [Pennie, 2000]. The use of DNA microarrays and RT-PCR to analyze the gene expression levels and profiles caused by pollutants has led to the detailed understanding of toxicogenomic mechanisms [Aardema and MacGregor, 2002]. Therefore, to investigate whether one can associate a specific gene expression to the exposure of some environmental toxicant and screen specific genes as biomarkers, it is necessary to concomitantly use both the DNA microarray and real time RT-PCR.

The main objective of this research is to study the environmental stresses of various toxic chemicals on *Escherichia coli, Saccharomyces cerevisiae* (Yeast), and *Oryzias latipes* (Japanese Medaka fish) through toxicogenomic analyses using both DNA chip and real time PCR. The outcome from this study can be further implemented in the development of environmental stress-responsive whole cell-based biosensors.

Materials and Methods

Bacterial Strains, Growth, Culture Medium and Luminescent Measurements

For the analysis of the gene expression patterns of *E. coli*, strain RFM443, which is the host strains for recombinant bioluminescent bacteria in author's laboratory, was used. Initially, 5ml cultures at an O.D. of 0.3 (600 nm) were exposed an aromatic compound, including phenol (451 ppm), 2-Cl-phenol (154 ppm), 2,4-Cl-phenol (24.7 ppm) and 2,4,5-Cl-phenol (4.7 ppm), or compounds known to induce DNA damage, such as MMC (50 ppb), or oxidative damage, such as hydrogen peroxide (10 mM) and paraquat (50 mM), for 1 hour. All DNA chip experiments used this protocol.

The recombinant bioluminescent bacteria, EBHJ (*sodA::luxCDABE*) [Lee and Gu, 2003], EBJM (*pqi-5::luxCDABE*), EBDO2 (*ompT::luxCDABE*), and EBDC1 (*clp::luxCDABE*), were constructed in this study. Each 0.1 ml culture was then exposed to each chemical and the bioluminescence (BL) was measured every 30 minutes using a Model 20e luminometer. After measuring the BL, the tubes were returned to the incubator until the next reading to maintain the temperature of the culture. The maximum BL ratio is defined in this study as the ratio of the maximum BL of the cells induced by each of the dioxin congeners to the maximum BL of the control cells, which were exposed to same concentration of the solvent alone.

Yeast Strain, Media, and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Saccharomyces cerevisiae S288C (ATCC26108) was used in the current study and grown in YM medium (DIFCO, USA) in 250ml flasks in a rotary incubator (Perkin-Elmer Co., USA) at 30°C and 200 rpm. Benzo[a]pyrene (B[a]P) was purchased from the Sigma Chemical Company. Specific primers were ordered for the corresponding nucleotide sequences. The yeast cultures were grown in YM medium after being inoculated with 2ml from an overnight culture. The B[a]P stock solution, prepared in benzene, was diluted into the media during the mid exponential growth phase so that the final concentration of benzene was 0.1 %, which did not result in any significant growth inhibition. Total RNA extractions were done 30 min, 45 min, and 60 min after exposure to 5µM B[a]P, with which no significant growth inhibition was seen, using the QIAGEN RNAeasy Mini Kit (#74104). The reverse transcription polymerase chain reaction (RT-PCR) was performed using a STRATAGENE ProSTR™ HF Single-Tube RT-PCR System under the following conditions: for reverse the transcription process - 15 min at 37°C and 1min at 95°C, and for the PCR - 30 sec at 95°C, 30 sec at 50 or 55°C and 1 min at 68°C with 25 reaction cycles [Lee and Gu, 2003]. The ACT1 gene, encoding actin mRNA, a commonly known housekeeping gene, was used as the standard for the RT-PCR.

Japanses Medaka Fish Culturing Methods

The adult fish of the orange-red variety of Medaka (*Oryzias latipes*) were maintained at the Environmental Toxicology Laboratory, National Institute of Environmental Research in Korea. They were reared under constant 18-h light:6-h dark cycles and a temperature of 25 ± 1°C. The Medaka used in this study was spawning females. The pH was controlled weekly and varied between pH 7-8. Fish were exposed in 1L glass beaker containing 600ml of dechlorinated local tap water. All of the chemicals used in this study were purchased from the Sigma Co., USA. 17β-estradiol and bisphenol A was dissolved in ethanol, while nonylphenol was dissolved in methylene chloride, and these were added to the aquarium water so that the solvent concentration in the aquaria was 0.006% [v:v]. Groups of control fish were exposed to the same solvent concentration. After the exposure ended, the total RNA was isolated from the liver of the exposed and control Medaka.

Hybridization of the Fluorescent-labeled cDNA and Statistical Analysis of the Gene Expression patterns

After exposure of the organisms, *i.e.*, bacteria, yeast or fish, with specific toxic chemicals, the total RNA was purified. The total RNA was labeled with Cy3 or Cy5 fluorescent dyes and all reactions involving these fluorophores were carried out in the dark as follows. After mixing 100μg RNA with 2μl oligo dT (2μg/μl), the mixture was incubated at 65°C for 10 minutes, chilled on iced, and then mixed with 9.6μl of a reverse transcription labeling mixture. This mixture consisted of 6μl 5×first-stand buffer, 3μl DTT (0.1M), 0.6μl dNTP mixture (25mM dGTP, 25mM dATP, 25mM dCTP and 10mM dTTP), 3μl Cy3-dUTP (control RNA) or Cy5-dUTP (treated RNA) (Amersham Life Sciences) and 2μl Superscript II reverse transcriptase. The resulting 30μl RNA mixture was incubated at 42°C for 2 hours. To degrade the template RNA, 15μl 0.1N NaOH and 0.1N HCl were added to the mixture, followed by incubation for 30 minutes at 65°C. The labeled single stranded cDNA (ss-cDNA) were purified using a PCR purification kit (Siege, #28104) according to the manufacturer's protocol, and then the cDNA labeled with Cy3 or Cy5 were mixed in one tube. Forty microliters of the hybridization mixture, containing 6.8μl 20×SSC (sodium chloride and sodium citrate), 1.2μl 10% SDS (sodium dodecyl sulfate), 2μl polyA RNA (10μg/μl, Sigma Co.), 2μl Yeast tRNA (10μg/μl, Gibco BRL) and 20μl Human Cot I DNA (1μg/μl, Roche Co.), were first incubated at 95°C for 2min, centrifuged briefly and then loaded on the cDNA chip. A cover slip was slowly placed over the solution. The slides were then incubated at 65°C for 14 hours in a water bath. Following the hybridization, the chips were first washed briefly in 2×SSC to allow the cover slip to gently come off, and then washed in 0.1×SSC/0.1% SDS at 50°C. In addition, the chips were washed twice in the same solution for 0.1×SSC for 5 minutes at room temperature and then dried by spinning at 600 rpm for 5 minutes.

The chips were scanned on a GenePick 4000 scanner (Axon Instrumnets Inc.) with a photomultiplier tube setting of 780V for Cy5 and 600V for Cy3. For data analysis, the local background was subtracted from the raw signal for each spot to generate the net signal. Spots marked with a "Flag" by the software, indicating the signal was too weak to make a call, were excluded. The Cy5/Cy3 net intensity ratios were normalized using the median to compare the ratios between the arrays.

Results and Discussion

Analysis of Toxic Effect in the *E. coli* cDNA Chips to the Environmental Toxicants

The cDNA chips used in this study contain 96 different genes, which are mostly span the sigma factors and SOS regulon, detoxification, and other metabolic pathway related genes of *E. coli*. *E. coli* strain RFM443 was treated with phenol, 2-Cl-phenol, 2,4-Cl-phenol, 2,4,5-Cl-phenol, mitomycin C, hydrogen peroxide, or paraquat, after which the total RNA was isolated from both treated and untreated cells. Figure 1 (a) shows that the expression patterns of the induced or down-regulated genes for each chemical are different. For the treatment with chlorophenols, *malE*, *malK* and *tnaA* (group C in figure 1 (a)) were highly induced. MalKFGE is a maltose transport system that is a member of the ATP-Binding Cassette (ABC) superfamily of transporters. MalE is a periplasmic maltose-binding protein and MalK is the ATP-binding component of the ABC transporter. A 10-fold increase in the level of maltose transport activity was observed in membrane vesicles when the membrane associated components of the transport system (MalF, MalG, and MalK) were overproduced. This means phenolic compounds may inhibit carbon source transport from the outer membrane to the inner. Mitomycin C especially affected the expression of *dinI, polB, recA, uvrA*, and *sbmC* (group D). These genes are related to DNA repair and replication, which fits the characteristic nature of Mitomycin C, which covalently binds the minor groove of DNA, thereby blocking replication. For hydrogen peroxide, the *sdhA, aldA, dadX*, and *hemH* genes were especially induced (groupB). Except for HemH, all the

other genes produced are dehydrogenases. As well, this chemical induced the *soxR*, *soxS*, *sodA* and *katG* genes.

Although the cDNA chip used here has limitation in terms of the number of genes being studied, we can clearly identify that different chemicals result in different gene expression patterns while chemically similar compounds led to similar gene expression patterns (Fig. 1(b)). These results suggest that a cDNA microarray-based expression analysis might offer an efficient means for assessing chemicals, drugs or other materials that require toxicity screening and can discriminate between different classes of chemicals.
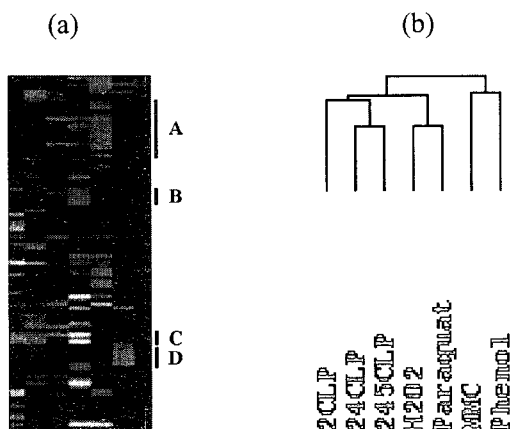
(a)   (b)

Fig. 1. Clustering analysis of different sets. (a) Gene expression patterns for the 7 different chemicals. (b) clustering gene expression patterns according to the chemical. (Correlation -centered), Clustering method-Average linkage)

As well, if all genes are assessed, we can select genes that are specifically up regulated or down regulated when the cells are exposed to certain substances. From this information, we can develop whole cell-based biosensors using specific genes and their promoters and bioluminescent or GFP reporter genes.

Analysis of Toxic Effects in *E. coli* Using Real-time PCR Analysis

The *recA*, *katG*, *fabA* and *grpE* genes in *E. coli* are specifically known to respond to environmental toxicants through an increased expression, and the expression of each gene is representative of DNA, oxidative, membrane and protein damage, respectively, as caused by environmental toxicants. By quantifying the expression levels of each of these genes after exposure with mitomycin C (MMC), hydrogen peroxide (H_2O_2), and phenol, it was found that *E. coli* selectively expresses these genes under such stressful conditions, indicative of specific responses related with the toxic effect each chemical results in. When the expression levels of each gene were compared for each compound, it was found that the expression of *recA*, representative of mutagenic effects, was in the greatest with MMC, and then H_2O_2 and finally phenol. On the other hand, the level of oxidative damage, based upon the expression of *katG*, was the highest with H_2O_2, followed by MMC and then phenol, while, for membrane and protein damage, phenol caused the greatest response and then H_2O_2 and then MMC. Therefore, the extent of each stress caused by these toxic chemicals can be evaluated by using the expressed level of these four genes.

(a)   (b)   (c)
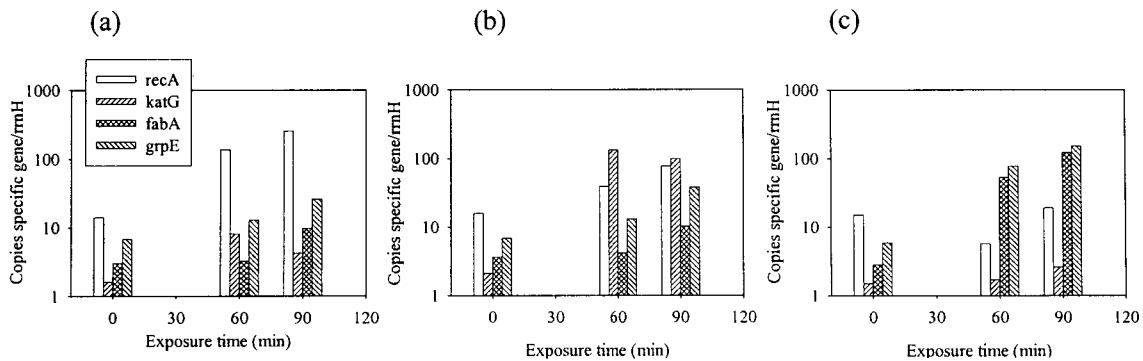
Fig. 2. Response patterns of the *recA*, *katG*, *fabA*, and *grpE* genes from *E.coli* strain REM443 after exposure 0.1ppm MMC (a), 0.0007% hydrogen peroxide (b), and 100ppm phenol (c) addition. All data is plotted relative the expression level of the 16S rRNA gene.

Effect of Benzo[a]pyrene on Genes Related to the Cell Cycle and the Cytochrome P450 Gene of *Saccharomyces cerevisiae*

Several genes of *Saccharomyces cerevisiae* were chosen as they are all conserved and critical in arresting each phase of the cell cycle [Lee and Gu, 2003]. To obtain relative transcriptional patterns, the RT-PCR products of the actin mRNA, encoded by the ACT1 gene, were used as the standard. Benzo[a]pyrene (B[a]P) is a ubiquitous carcinogen produced from the incomplete combustion of organic carbons. B[a]P itself is not known to cause any toxicity, but rather its degradation products.
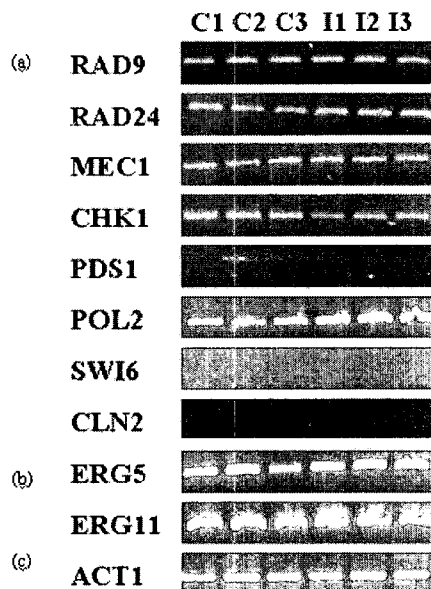

Fig. 3. Results of RT-PCR after exposure to 5μ M B[a]P. (a)DNA damage checkpoint genes and (b) cytochrome P450 genes. In addition, the ACT1 gene was used as the standard (c). C1, C2 and C3 are the results for the unexposed samples, while I1, I2 and I3 are the results for the samples exposed for 30, 45, and 60min, respectively.

The expression of the selected genes were not highly altered with the addition of B[a]P at sub-lethal concentrations, below which no significant growth rate inhibition was apparent. Only the POL2 gene, related to DNA synthesis, was induced. In addition, the decision for the cells to differentiate is known to be directly

dependent on the cell cycle arrest in the G1 phase. However, neither of the genes chosen due to their relation to a G1 arrest, the SWI6 gene, encoding a transcription factor for cyclins, and the CLN2 gene, one of the cyclin genes required to start the cell cycle, were induced (Fig.3a), indicating that B[a]P did not cause a G1 arrest of *S. cerevisiae* at the concentrations tested. Two cytochrome P450s that require CPR for their function and genes, CYP51 (erg11), encoding sterol 14α -demethylase, and CYP61 (erg11), encoding a sterol □ [22]- desaturase, were used to evaluate the stresses resulting from the addition of B[a]P to cultures of *S. cerevisiae*. As shown in Fig. 2b, the expression of CYP51 and CYP61 increased over time, yet decreased 45 min after the initial exposure to B[a]P. The experimental results also revealed that CYP51 was more highly induced than CYP61. Accordingly, the current study investigated the expression patterns of two categorized groups of genes, DNA damage checkpoint genes and cytochrome P450 genes, both of which are associated with carcinogenesis in higher eukaryotic systems, in the budding yeast, *S. cerevisiae*, in response to the addition of B[a]P. These genes may also be important when yeast experiences xenobiotic stresses, including exposure to B[a]P. Therefore, the present results indicate that cytochrome P450 enzymes seem to play a more critical role than DNA damage checkpoint genes in the response to and the metabolism of B[a]P in yeast.

Use of Functional cDNA Chips in the Analysis of Effects in Japanese Medaka Fish Resulting From Exposure to Environmental Toxicants

In this study, the expression levels and patterns of 21 different stress responsive genes after treatment with 17β-estradiol, nonylphenol, bisphenol A, known EDCs, were analyzed using a fish cDNA microarray.

(a)

(b)
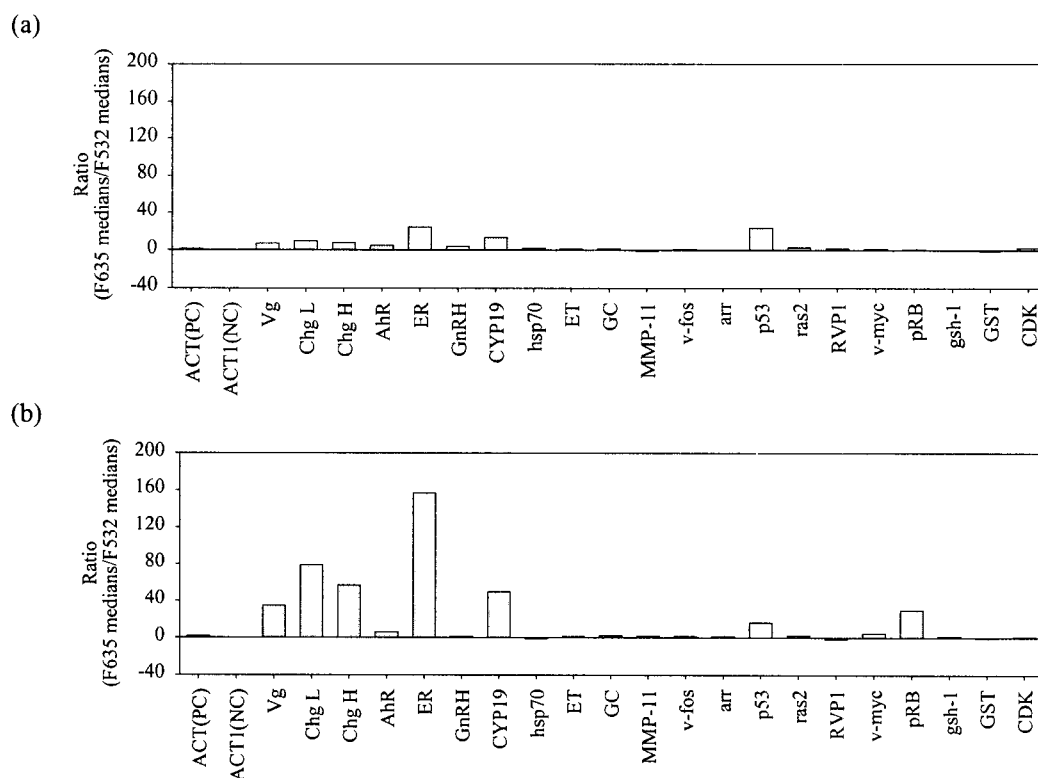

Fig. 4. DNA microarray analysis of the female liver mRNA expression levels of Japanese Medaka fish after being exposed to 100μg/l 17β-estradiol. Results from a (a) 1-day and (b) 10-day exposure.

Female Medaka fish were exposed to 100μg/l of 17β-estradiol for 1 and 10 days, (Fig.4), and the livers were harvested and the purified RNA samples were used to analyze the expression levels of the genes. As shown in Fig.4a, of the 21 stress responsive genes, 8 genes were up-regulated, *i.e.*, vg, ChgL, ChgH, AhR, ER

GnRH, CYP19 and p53, with a short exposure time (1 day), but all were induced less than 20-fold when the fluorescence of the exposed samples were compared to those of the control. On the other hand, after a 10-day exposure, the expression levels of vg, ChgL, ChgH, ER and CYP19 were much higher than those seen for a 1-day exposure, while those of the AhR and p53 genes did not change (Fig.4b). In addition, an exposure to 17β-estradiol for 10 days results in the induction of pRB, which encodes for retinoblastoma, a control for the onset of cellular senescence.

Comparison of Functional cDNA Chip Analysis with the Real Time PCR Analysis of Japanese Medaka Fish Exposed to EDCs

The effect of EDCs on Japanese Medaka in this study is fundamentally reflected at the cellular level by its impact on expression of three genes, *i.e.,* the estrogen receptor (ER), cytochrome P450 aromatase (CYP19) and p53 genes. Consequently, measurement of the transcriptional (mRNA) levels of the three genes will reveal valuable information about the potential toxicity of EDCs before the development of a toxic or pathological response in the fish. In this study, two important methods available for the study of gene expression at the transcriptional level, *i.e.,* DNA microarrays based on hybridization techniques and quantitative (real time) RT-PCR based on PCR techniques, were performed to determine the early signs of toxicity caused by particular EDCs in Medaka. As shown in Fig.5, the differential RNA expression patterns of certain genes, through the use of DNA microarrays and real time RT-PCR, provide some insights into the potentially different toxic actions of the EDCs, *i.e.,* 17β-estradiol, nonylphenol and bisphenol A, in the fish. In addition, non-EDCs, such as phenol and glyphosate, were found to result in RNA expression patterns that were different when compared with those of the EDCs. Real time RT-PCR has been widely used for measuring differential gene expression and for validating data from DNA microarrays. To compare the gene expression patterns between the DNA microarrays and real time RT-PCR, Fig.5 has been prepared. It compares the ER, CYP19 and p53 gene expression levels using both the DNA microarray and real time RT-PCR. As can be seen, the overall trend between the two methods is strikingly similar, although the absolute fold-induction was not same. However, the results indicate that all three genes are regulated in the same manner in response to the three EDCs, further indicating that these EDCs exert toxicity through similar mechanisms.
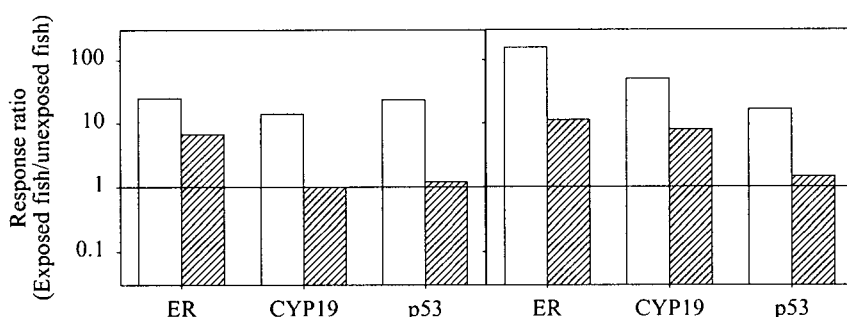
Fig. 5. Comparison of DNA microarray and real time RT-PCR. The response ratio for RNA levels in exposed fish divided by unexposed fish was determined by array analysis and real time PCR from the same samples and is shown for the three genes with the most differential response to the 100μg/l of 17β-estradiol, which is the EC20, obtained from acute toxicity assays. Open boxes: DNA microarray data. Shaded boxes: real time RT-PCR data. Left column: 1-day exposure. Right column: 10-day exposure.

Concluding remarks

Bioluminescent or fluorescent cells have been used in the classification and characterization of numerous substances and their toxicities, including PAHs, pesticides, dioxin congeners, and exposure to ionizing radiation. However, these cells were developed based upon the results of traditional time consuming gene expression analysis, random priming or shot-gun based trail and error searches, with each gene to be studied needing to be cloned one by one. With the introduction of DNA microarrays and real time RT-PCR techniques, though, a high throughput analysis of stress-induced gene expression is now possible and is being applied in stress analysis since we need to know the mechanisms of how cells respond to toxic chemicals on the genetic level. Therefore, the development of biosensors is about to enter a new generation using the plethora of knowledge about gene expression patterns obtained with DNA microarrays and real time RT-PCR. Based upon these techniques, our research groups have developed and will continue developing new recombinant strains that can respond to toxic chemicals and conditions. This new era of molecular biology and techniques will lead to a explosion in the number of recombinant strains developed.

Acknowledgments

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