BIOMARKERS, 1999, VOL. 4, NO. 2, 93–105

REVIEW

Molecular approaches to identify exposure and risk to specific environmental pollutants

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Received 17 June 1998, revised form accepted 13 August 1998

Ecotoxicology and environmental epidemiology require an intimate understanding of the relationship between exposure of a population to a pollutant and the subsequent biological effects. This article summarizes two relatively new and powerful techniques to examine both exposure and response to environmental pollutants which build upon an increased understanding of how particular classes of chemicals elicit toxicity at the molecular level. The genetic reporter assay can be used to examine the exposure and potential toxicity of environmental samples. Quantitative polymerase chain reaction (PCR) is an exquisitely sensitive and adaptable procedure to examine early events, i.e. biomarkers, that might be indicative of exposure and/or sensitivity to a chemical insult. Taken together, these assays are important new tools in assessing the exposure and risk of human and wildlife populations to such important pollutants as dioxins, PCBs, oestrogenic and anti-oestrogenic compounds, phthalate esters, DDT metabolites and heavy metals.

Keywords: polymerase chain reaction (PCR), molecular biomarkers, gene expression, reporter assays, environmental pollutants.

Abbreviations: AhR, aryl hydrocarbon receptor; AP, alkaline phosphatase; β-Gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; MT, metallothionein; OR, oestrogen receptor; PAH, polycyclic aromatic hydrocarbons; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase polymerase chain reaction.

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Introduction

The relationship between exposure of a population to a pollutant and the subsequent biological effects is an important aspect of ecotoxicology and environmental epidemiology. Many of the current studies assessing the risk of pollutant exposure in wildlife and human populations involve the measurement of specific chemical residues present in sediment, water or soil that the population habituates or of those chemicals that have accumulated in its tissues. Subsequently data from a similar species or a laboratory animal are used to predict the toxicological impact of this exposure (Dickerson *et al.* 1994). In addition to being quite expensive and laborious, chemical residue analysis is often not a good indicator of bioavailability or the ability to induce toxicological responses (Dickerson *et al.* 1994). The use of relevant biological markers ('biomarkers') can

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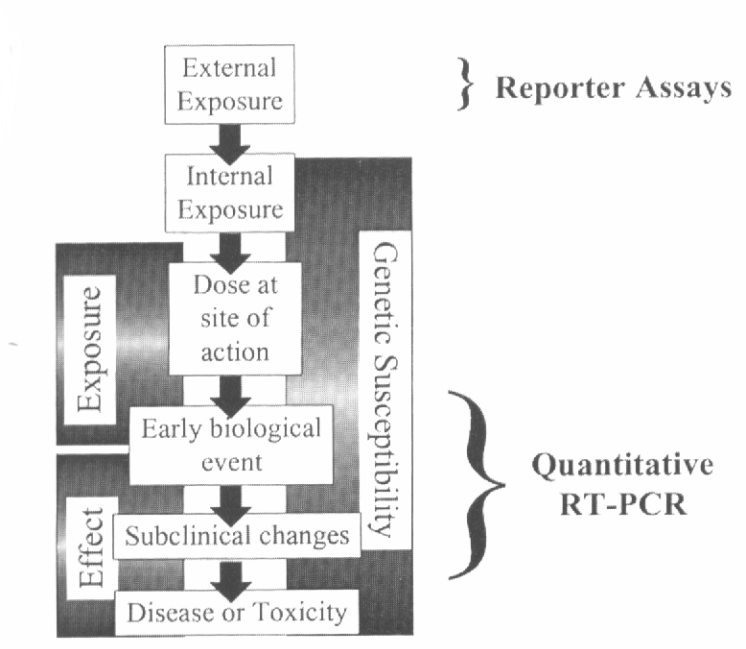

Figure 1. The toxicological paradigm. The sequence of events initiated by chemical exposure and resulting in an adverse response is deemed the 'toxicological paradigm'. Biomarkers may be used to assess key points in the paradigm and include exposure, effect and susceptibility. Reporter assays and RT-PCR may be utilized to assess different aspects of this sequence, as indicated.

overcome these limitations and may provide important information for use in ecological risk assessment.

A biomarker is defined (NRC 1989) as a 'xenobiotically-induced variation in cellular or biological components, processes, structures, or functions that are measurable in a biological system or samples'. When identifying a putative causative agent it is helpful to think of the relationship between exposure and disease as a multistage process which includes external exposure, internal dose, early biological effects, altered structure and function and finally clinical changes (Links *et al.* 1995). In characterizing relationships between a xenobiotic and a toxicological response, it is desirable to distinguish each step, if possible, in this paradigm. Biomarkers signify these alterations in biological systems and may be indicators of exposure, dose, effect or susceptibility (Links *et al.* 1995; see figure 1). A marker of *exposure* or *dose* may be an exogenous compound or metabolite within the body, or an interactive product of the compound and an endogenous compound. The second type of biomarker, that of *effect*, is a measure of function or balance that is affected by the exposure and is usually, but not always, subclinical. The third type of biomarker is that of genetic *susceptibility* and it permeates all aspects of the toxicological paradigm. A susceptibility biomarker may be inherited and if induced will indicate if an individual is more sensitive to xenobiotic exposure (Grandjean *et al.* 1994). In the present article, we will address the use of genetic reporter assays as a biomarker of potential *exposure* and altered gene expression as an indicator of *effect*.

The development of effective molecular biomarkers for environmental agents is based on several factors (outlined in Dickerson *et al.* 1994). First, the effect being studied should be indicative of exposure and, if known, the biological or

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toxicological effect of a chemical. Therefore, the chemical should exhibit quantitative, and predictable, dose–response relationships. Second, the biomarker examined should be specific for a particular chemical or class of compounds. This is only possible if one understands the molecular mechanisms by which the chemical is acting. Last, in an ideal case the biomarkers should be relatively non-invasive and compatible with the protection and conservation of the organism being examined (Fossi 1994). Other considerations include the cost of performing the analysis and the ability to examine archived samples.

The purpose of the present article is to summarize two state-of-the-art methods to examine both exposure and response to environmental pollutants. The use of reporter assays to examine external dose and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to measure early biochemical events (figure 1) rely heavily on mechanistic information about particular groups of chemicals. In particular these methods are well suited for chemicals that affect the transcription of genes via interaction with a specific intracellular protein or receptor (Sewall *et al.* 1995). The types of chemicals that fit into this category include, but are not limited to: polycyclic aromatic hydrocarbons (PAHs) such as dioxins and PCBs acting through the aryl hydrocarbon receptor (AhR); oestrogenic and anti-oestrogenic compounds such as the insecticides kepone and dieldrin acting at the oestrogen receptor (OR); phthalate esters and DDT metabolites which activate the peroxisome proliferator-activated receptor (PPAR), and heavy metals such as cadmium which induce gene transcription through the metallothionein (MT) protein. The basic theory behind each essay will be described and specific examples given that supports its use in molecular epidemiology or ecotoxicology. As will be shown, both techniques meet the criteria put forth for the development of effective molecular biomarkers for exposure to environmental agents.

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Quantitative RT-PCR as a tool to examine biomarkers of exposure and effect

Basics of quantitative RT-PCR

The polymerase chain reaction (PCR) is an enzymatic assay which is capable of producing large amounts of a specific DNA fragment starting from a small amount of a complex mixture (figure 2, reviewed in Mattes 1997). In the case of RT-PCR, the messenger RNA must first be converted to a double stranded molecule with the use of the enzyme reverse transcriptase. The thermostable DNA polymerase (i.e. Taq) and the use of specific 'primers' are the key features of any PCR reaction. All known DNA polymerases require deoxyribonucleotide triphosphates (dNTPs), a divalent cation (Mg^{2+} or Mn^{2+}), a DNA or cDNA template, and a region of that template that is double-stranded adjacent to a single stranded nick or gap. The double-stranded region is provided by the primer annealing to its complementary region of the DNA template. If the starting mixture includes not only a single stranded polynucleotide template, but also: (a) its complementary strand, and (b) two oligonucleotide primers that hybridize to both strands, copies of *both* of these strands will be produced each cycle and these copies can be used as templates for subsequent cycles. Short DNA fragments whose ends are defined by the position of the two oligonucleotide primers will accumulate in an exponential fashion, i.e. like a chain reaction. If 30 cycles of PCR are performed, theoretically one will achieve a 2^{30} amplification of the starting biomarker cDNA. The product which is formed is specific for a particular biomarker as dictated by the design of the oligonucleotide primers.

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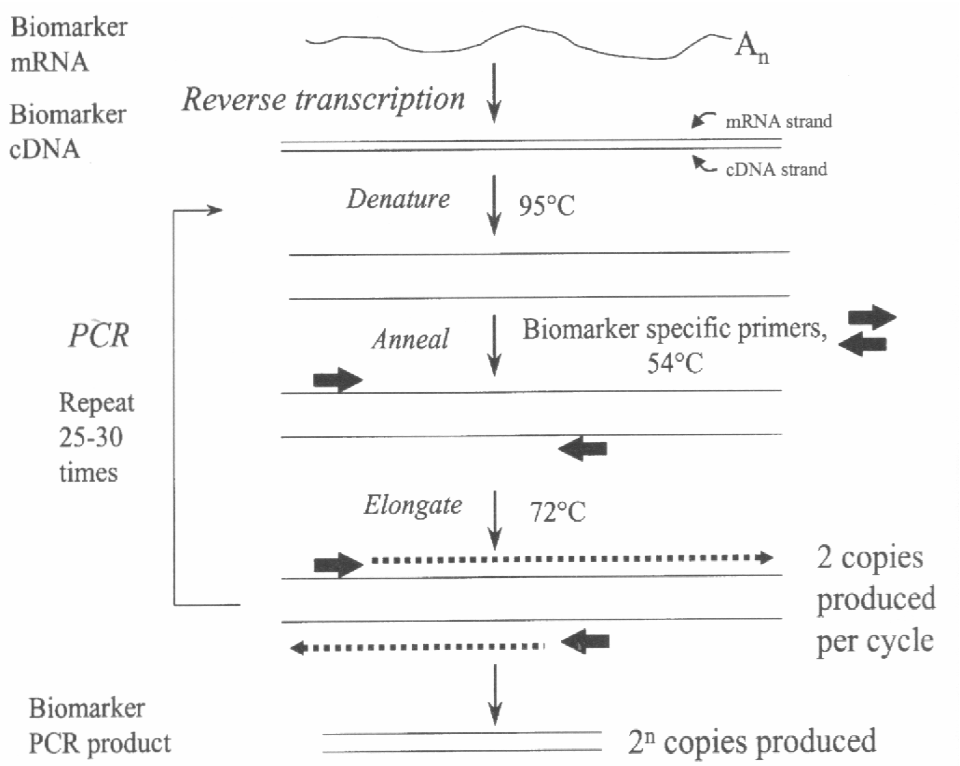

Figure 2. Basics of reverse transcriptase polymerase chain reaction. See text for details.

Therefore, RT-PCR is a tool to examine the messenger RNA expression of a xenobiotic-inducible gene with the amount of product formed a function of the amount of starting template. Of course, the examination of mRNA accumulation can be determined in many cases by hybridization procedures such as Northern blots, dot- or slot-blots, and RNase protection assays. Nonetheless, in terms of amount of sample required, detection of small differences in expression and ability to examine many genes in a large number of samples, RT-PCR stands above the more conventional procedures. However, there is a negative aspect of using RT-PCR to quantify mRNA levels as a biomarker. That is, an internal standard (IS) is necessitated in these assays due to the fact that there is a large amount of tube-to-tube variability in amplification efficiency. For example if 10 tubes of seemingly identical reagents are PCR amplified, there could be as much as a three-fold difference in the amount of product formed. If an IS was co-amplified with the target, the efficiency of amplification in each tube could be corrected and this three-fold difference could easily be negated.

There are numerous considerations to making a good internal standard for quantifying mRNA levels, but the discussion of this process is beyond the scope of the present article. The reader is directed to other sources (Vanden Heuvel 1997) for a description of IS construction methods and considerations. In addition, once an IS is produced or obtained, the investigator has several options as to which method to use for the actual RT-PCR assay. However, in general, the 'competitive' RT-PCR approach is the easiest and most adaptable procedure. The standard competitive RT-PCR approach is shown in figure 3 (adapted from Gilliland *et al.* 1990). The basis for this method is that the more competitor (i.e. IS) that is

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present, the less likely the primers and Taq DNA polymerase will bind to the biomarker cDNA and amplify. Therefore, despite the fact that all reagents are there in extreme excess (i.e. primers, enzyme, $MgCl_2$, nucleotides) the reaction appears to be competitive. That is, as the amount of competitor is increased, less and less biomarker PCR product is formed until eventually only the IS product is observed. When a dilution series of IS is spiked into a constant amount of RNA, it is possible to estimate the amount of a specific product present in the sample. As depicted in figure 3, an increase or decrease in biomarker mRNA is easy to visualize using competitive RT-PCR. In addition, calculation of amount of biomarkers mRNA in each sample is quite facile if one has access to standard laboratory photographic and densitometric equipment (Vanden Heuvel 1997). In general terms, the amount of IS required to result in a 1:1 ratio of IS:biomarker PCR product is representative of the amount of biomarker present in the original sample (i.e. in figure 3, 10^6 versus 10^7 molecules biomarker mRNA in total RNA derived from control- and chemically-exposed animals, respectively). As little as a two-fold difference between exposure groups can be routinely identified using a very small amount of RNA (i.e. 10–100 ng total RNA, the equivalent of approximately 1000–10 000 cells).

Choosing an appropriate biomarker

As stated above, the choice of effective molecular biomarkers for environmental agents is based on several factors. The biomarker being studied should be indicative of exposure and the biological or toxicological effect of a chemical, exhibit quantitative dose–response relationships and be specific for a particular chemical or class of compounds. In the case of receptor-mediated xenobiotics, one should identify genes whose mode of regulation is well characterized. The accumulation of a biomarker as the result of chemical exposure may be the result of many mechanisms including altered mRNA transcription, processing and stability; if an enzymatic assay is being studied translation efficiency, post-translational modification, stability and co-factor levels may be affected by a particular xenobiotic. In addition, genes may be regulate through non-receptor mediated events, including stress-related responses. Examining *mRNA levels* of a gene that is controlled at the *transcriptional level* by a *xenobiotic receptor* is the least complicated, and hence the easiest to interpret, biomarker available.

There are many examples of biomarkers which fit the criteria stated above. Interestingly, often this biomarker is a member of the xenobiotic-metabolism pathway (cytochrome P450, glucuronyltransferases, esterases, multi-drug resistance). Teleologically, the organism's primary response to a toxicant, such as a dioxin or PCB, may be to try to increase the removal of the foreign chemical. Therefore, the subtype (isozyme) of a metabolic enzyme induced by a chemical may be specific for that particular class of chemical. Note that many stress-related biomarkers (i.e. heat shock proteins; De Pomerai 1996) are indicative of an insult to the animal, however, they are not specific for the class of chemical being examined. The same may be said for DNA damage response genes such as GADD45 or p53 which may be representative of chemical insult, but cannot be used to determine the source of the damage. Examples of good molecular biomarkers, i.e. induced at the transcriptional level by a specific xenobiotic receptor, are given in table 1. As shown in figure 4, the induction of mRNA for cytochrome P450s CYP1A1 and

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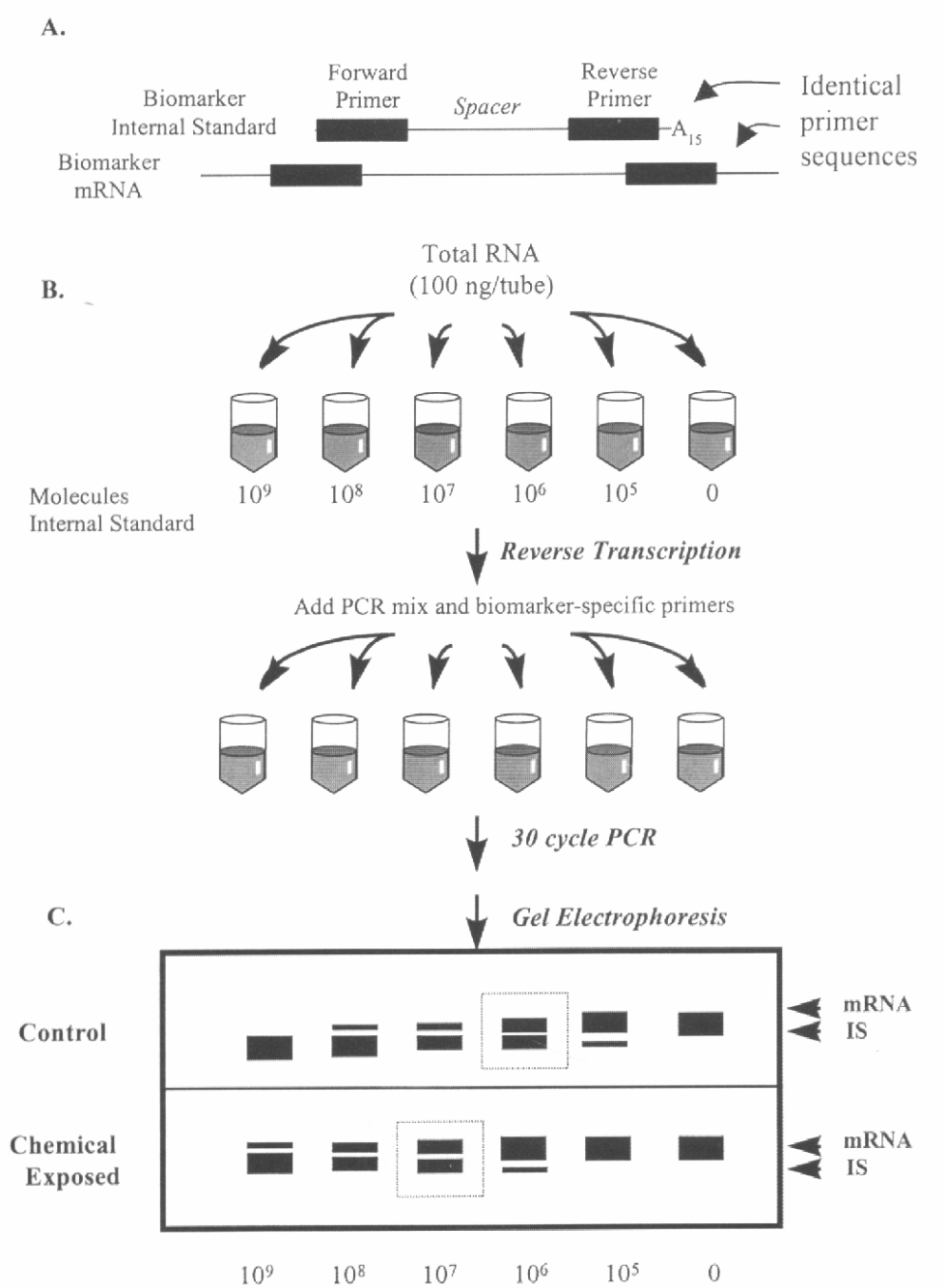

Figure 3. Quantitation of gene expression by RT-PCR. The concentration of mRNA for molecular biomarkers can be effectively evaluated using a competitive RT-PCR technique, as depicted. Panel A: representation of the RNA internal standard (IS) in contrast to the target (biomarker) mRNA. The forward and reverse primer sequences on each are identical, although the spacer placed in the rcRNA results in a PCR product which can be easily resolved from biomarker mRNA. Panel B: general RT-PCR reaction using a constant amount of RNA (100 ng per tube) with varying amounts of internal standard ranging from 10^9 to 10^5 molecules per tube. Panel C: representative photograph of biomarker mRNA analysis from control and exposed individuals. Note the increased biomarker: IS ratio in the PCR products form the exposed individual, clearly demonstrating the induction of gene.

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Table 1. Molecular biomarkers appropriate for examining the exposure and responsiveness to particular xenobiotics.

Chemical class (receptor system)	Possible molecular biomarker
Dioxins, co-planor PCBs, PCDFs (AhR)	Cytochrome P4501A1 (CYP1A1)
Peroxisome proliferators (PPAR)	Cytochrome P4504A1 (CYP4A1), acyl-CoA oxidase (ACO)
Oestrogenic and anti-oestrogenic chemicals (OR)	Vitellogenin
Heavy metals (MT)	Metallothionein IIa

CYP4A1 are excellent biomarkers for exposure to AhR ligands (dioxins, PCBs) and PPAR activators (phthalate esters), respectively. Also note that the extent of induction is substantial (> 10 000 fold induction) and that the method is exquisitely sensitive. For example, the induction of CYP1A1 mRNA is the most sensitive marker yet described for dioxin exposure (Sewall *et al.* 1995).

The data in figure 4 clearly demonstrate that mRNA accumulation in a laboratory animal is indicative of the dose given, thereby meeting one of the criteria set forward for an effective biomarker. In addition, CYP1A1 mRNA accumulation can be measured in human peripheral lymphocytes, using the PCR procedures described above (Vanden Heuvel *et al.* 1993). Certainly, these non-destructive collection procedures may be used in other species as well, although the exceeding small samples obtained may preclude the use of most non-molecular, biomarkers. CYP1A1 cDNA has been cloned, and induction of this gene shown, in several wildlife species (Ashely *et al.* 1996) such as fish, crayfish, mink, otters and birds exposed to polycyclic aromatic hydrocarbons. For example, sea and river otters exposed to the oil in the Prince William Sound have a significantly higher expression of CYP1A1 mRNA than do non-exposed counterparts (P. Snyder, Purdue University, personal communication), indicative of PAH accumulation in

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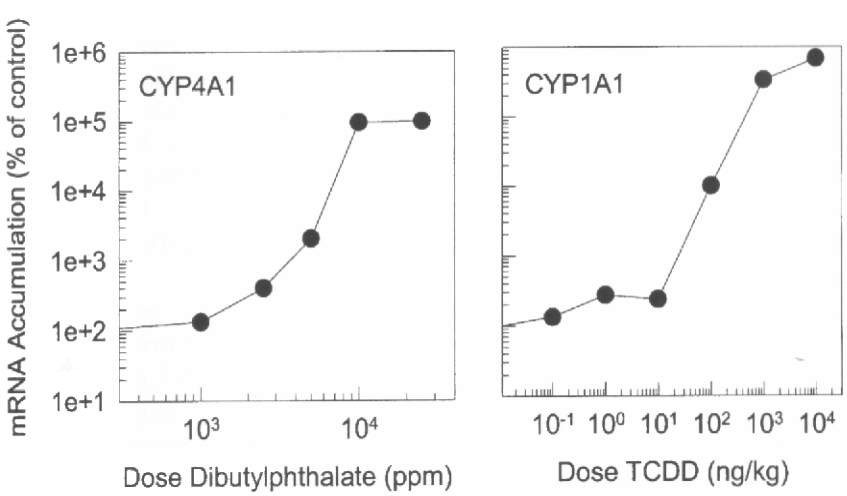

Figure 4. Representative dose–response relationships for induction of biomarker mRNA by xenobiotics. Left panel: the induction of cytochrome P450 IVA1 (CYP4A1) by the plasticizer and peroxisome proliferator dibutylphthalate in rat liver. Right panel: accumulation of cytochrome P4501A1 (CYP1A1) mRNA by 2,3,7,8 tetrachlorodbenzo-*p*-dioxin in rat liver. Both genes were examined using competitive RT-PCR as described above. The data points represent the mean of 5–10 animals.

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the liver. Mallard ducks exposed to the petroleum component benzo[*a*]pyrene also show higher CYP1A1 mRNA levels (M. Wolfe, University of California-Davis, personal communication). These studies both took advantage of RT-PCR techniques to measure CYP1A1 mRNA as a biomarker of effect. The sensitivity, specificity and ease of use of RT-PCR analysis makes it a prime candidate for performing these measurements. Low level exposure of a variety of animals (humans and wildlife), to a mixture of different chemicals can be evaluated in a non-destructive manner. In addition, archived tissue samples may be examined retrospectively.

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Assessing exposure to xenobiotics using reporter assays

Overview of genetic reporter systems

In recent years, the field of toxicology has moved toward more molecular techniques to investigate the response of cells to various xenobiotics. While data using whole animals are useful, understanding the cellular mechanism by which chemicals act is crucial in helping scientists assess the risk of exposure to chemicals. At the molecular level one of the most common and relevant endpoints of study is the alteration of gene expression (Todd *et al.* 1995). Assessing changes in gene expression in tissues is accomplished by a variety of methods including RT-PCR (discussed above) and Northern blotting. On the other hand, if one were interested in determining the *potential* for toxicity of a pure chemical or an *environmental mixture*, then reporter assays represent a fast and simple alternative (Kain and Ganguly 1996).

The principles which underlie genetic reporter assays are quite simple but require familiarity with some basic molecular and cellular biology concepts (see figure 5). First, one must have an appropriate cell model, usually a transformed cell line, which can be easily maintained in culture. This cell line is manipulated by incorporating, or transfecting, exogenously supplied DNA. Transfection of exogenous genes into mammalian cells is one of the most commonly used methods for analysing mammalian gene expression *in vivo* (Kain and Ganguly 1996). The genes that are incorporated, through the use of plasmid vectors, are:

(1) The receptor or transcription factor which responds to the exposure of an exogenous chemical ('expression vector'). The plasmid containing the genetic information for the oestrogen receptor or metallothionein, for example, also contains sequences which allow for high level expression within the model cell system.

(2) A reporter, i.e. gene that is regulated by the xenobiotic-responsive receptor or transcription factor ('reporter vector'). The actual reporter protein must be easy to detect and quantitate, and must contain sequences of DNA (promoter region) that are recognized by the product of the expression vector, as discussed below.

Regardless of the reporter plasmid utilized, the fusion gene usually consists of the binding site or enhancer sequence for the xenobiotic-responsive protein, a promoter sequence, and finally the coding region of a reporter molecule (Alam and Cook 1990). In these experiments two assumptions are generally made. One, the amount of the reporter protein translated is presumed to reflect the ability of the chemical to activate the product of the expression vector. This is true only if the activated protein recognizes the specific enhancer/promoter region of the reporter

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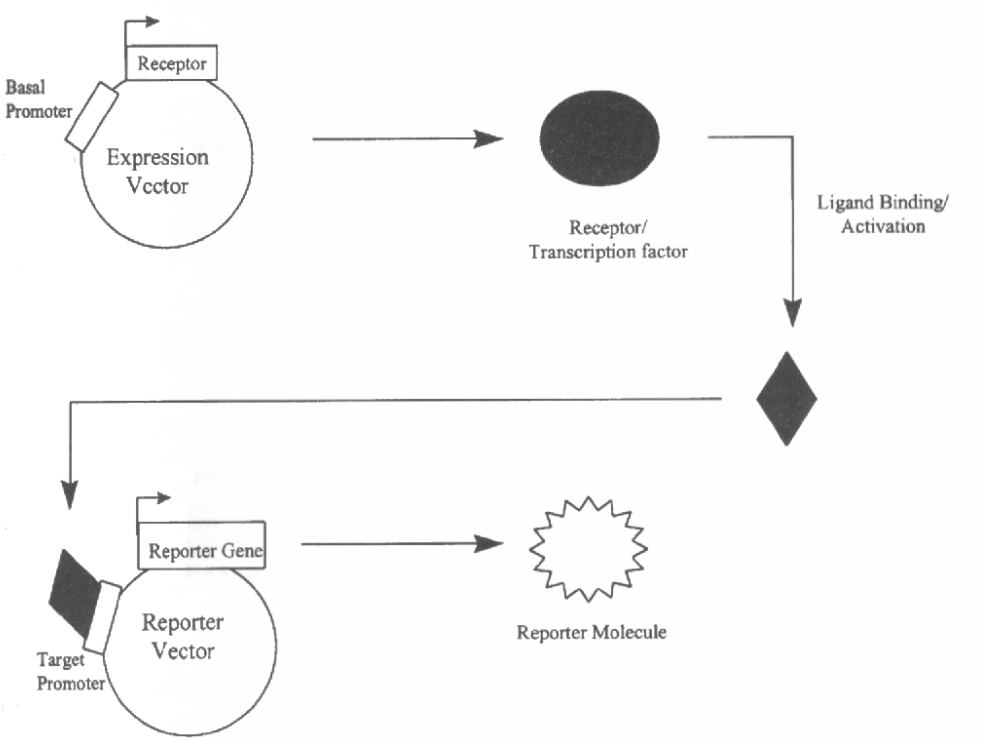

Figure 5. Mammalian cell transfection and reporter assays. Mammalian cells are co-transfected with plasmids encoding a functional receptor or transcription factor driven by a basal promoter and a reporter vector consisting of a target promoter or responsive element inserted upstream of a gene that encodes a reporter molecule. Upon activation the expressed receptor binds to the *cis*-element and regulates the expression of the reporter molecule.

construct and regulates the transcription of the reporter protein. Second, the reporter molecule has minimal effects on the physiology of the transfected cell model system. With all of the major genetic reporter assays used, this assumption is not a major concern.

There are many reporter systems from which to choose. The ideal system would be one that utilizes a reporter protein that is easy to quantify, sensitive, retains linearity of response over a wide range of expression, and is non-toxic to the host cell (Kain and Ganguly 1996). The most popular reporter systems are those that employ the chloramphenicol acetyltransferase (CAT), alkaline phosphatase (AP), firefly luciferase or β-galactosidase (β-gal) plasmids. Recently, another system has been developed and is gaining favour, the green fluorescent protein (GFP) system (Chalfie *et al.* 1994). The CAT assay is usually an isotopic-based detection system that is simple to use, but lacks sensitivity. Both β-gal and AP are colorimetric assays. Luciferase employs a chemiluminescent-based assay. This latter system has advantages over the CAT assay in that it is non-isotopic and quite sensitive, which is a particular advantage when using cells that are difficult to transfect. Finally, GFP, cloned from the jelly fish *Aequorea victoria,* uses a fluorescent tag that requires no co-factors or substrates. Regardless of the type of plasmids that are utilized, the amount of reporter detected must be proportional to the amount of xenobiotic applied to the cells and requires a fair amount of optimization and standardization.

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Table 2. Commonly employed genetic reporter plasmids[a].

Promoter-fusion construct	Endogenous gene product
CYP1A1	*Cytochrome P4501A1*
GST Ya	Glutathione S transferase Ya subunit
HMTIIA	*Metallothionein$_{IIA}$*
FOS	c-fos
XFH	Collagenase
HSP70	70-kDa heat shock protein
GADD153	153-kDa growth arrest and DNA damage protein
GADD45	45-kDa growth arrest and DNA damage protein
GRP78	78-kDa glucose-regulated protein
Response element-fusion construct	Name
OR	*Oestrogen receptor response element*
XRE	*Xenobiotic (aryl hydrocarbon) response element*
NκBRE	NF–κB response element
CRE	cAMP response element
P53RE	53-kDa protein tumour suppressor response element
RARE	*Retinoic acid response element*
PPRE	*Peroxisome proliferator-activated receptor response element*

[a] Responses that are indicative of a specific class of chemicals are shown in *italicized* type. The remaining reporter plasmids are used to examine a more generalized, stress-related response.

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Uses of genetic reporter systems in toxicological screening

Historically, the principal endpoint in classical toxicology has been histopathology. This method suffers from being laborious and insensitive and does not take into account key, and sensitive, biological endpoints (MacGregor *et al.* 1995). Also, the data gathered by these techniques do not shed any light on the *mechanism* of action of environmental toxicants or the *potential* for toxicity of a particular exposure. The development of reporter assay systems has led researchers to suggest a separate branch of toxicology, i.e. functional toxicology where chemicals are defined more by their mechanism of action than by their chemistry (McLachlan 1993). This strategy involves the use of cell culture models that are stably transfected with the molecular constructs discussed above for specific receptors, transcription factors and reporter genes, the idea being that if a panel of receptor-containing cells were designed, one could screen environmental samples of unknown biology or toxicology and determine their ability to result in gene activation (McLachlan 1993). Fortunately, the mechanisms by which very important classes of chemical alter gene expression have been characterized and are at the disposal of researchers interested in ecotoxicology (see table 2). The types of responses that can be examined are chemical-class-specific (i.e. the receptor-mediated xenobiotics) or a generalized indicator of chemical insult (i.e. stress response).

This reporter gene approach has proven to be especially useful in evaluating the presence of xenobiotics that result in toxicity through their ability to bind to endogenous receptor proteins. As mentioned above, this includes polycyclic aromatic hydrocarbons (PAHs), oestrogenic and anti-oestrogenic compounds, phthalate esters and DDT metabolites and certain heavy metals. For example this approach has been used to determine if environmental samples contain chemicals with oestrogenic or anti-oestrogenic activities. Environmental-oestrogens can be defined as any compound that can bind to an oestrogen receptor (OR) and elicit or modulate an oestrogen-mediated response (Balaguer *et al.* 1996). In many cases

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these chemicals share little or no structural similarities to traditional OR ligands such as 17β-oestradiol (see McLachlan (1993) for examples). In addition to pure chemicals, complex environmental mixtures can be evaluated in a similar manner. Balaguer *et al.* (1996), stably transfected HeLa cells with a Gal4-human OR chimeric construct and a Gal4-regulated luciferase reporter gene. Using this cell model, Balaguer and co-workers examined the ability of pulp and paper mill black liquor and urban air particulates to bind to the OR construct and induce expression of the reporter. They reported that the black liquor mixture was able to elicit a maximal response of 65% of the maximal response induced by 17β-oestradiol. Furthermore they demonstrated that this induction was suppressed by the anti-oestrogen, ICI 164,384. The urban air particulate matter did not induce expression of the reporter. However, when tested in a system that contained a dioxin responsive element the luciferase gene was induced. Similarly, Vincent *et al.* demonstrated that induction of reporter genes that are responsive to xenobiotics (CYP1A1, GSTYa, and XRE) was consistent with the detection of polycyclic aromatic hydrocarbons (Vincent *et al.* 1997). Also, the estimates of metal availability in these samples correlated with the magnitude of induction of the transgenes that are responsive to metals, i.e. HMTIIa and HSP70. These studies indicated the presence of oestrogen- or dioxin-like chemicals and heavy metals in environmental samples. Since we have a basic understanding of how these chemicals elicit their effects, exposure of human or wildlife populations to these mixtures could result in a fairly predictable sequence of toxic responses.

Besides directly examining receptor-mediated events as above, there are a battery of damage-inducible genes or generalized stress genes. The products of these genes play a role in the cellular response of a given toxicant either by detoxifying it, transporting it out of the cells, by repairing the damage it causes to cell macromolecules, or by intercepting toxic intermediates (MacGregor *et al.* 1995). Stress genes can be induced by many types of cellular damage such as lipid oxidation, DNA damage, osmotic imbalance, protein misfolding, disruption of electron transport, and membrane permeability, and hence are influenced by a wide array of environmental chemicals. Many mammalian stress promoters and response elements have been cloned and characterized (reviewed in MacGregor *et al.* 1995). Xenometrix (Boulder, CO) has developed a human liver cell assay in which promoters or response elements are fused immediately upstream of the CAT structural gene. Each of these constructs are stably integrated into the HepG2 cell line (see Todd *et al.* 1995 and table 2). Xenometrix has designated this the CAT-Tox (L) Assay. since 14 different transcriptional responses can be measured simultaneously under identical conditions, this system can provide stress profiles for a variety of toxic compounds. Each specific profile can then be used as a toxicological fingerprint and can be used to identify specific compounds in a complex environmental mixture. The CAT-Tox (L) Assay has been used to evaluate the presence of stress-inducing toxicants in suspensions of paticulates from ambient air and environmental samples taken from the Great Lakes Basin (Vincent *et al.* 1997).

Conclusion

The evaluation of a potential health risk due to exposure of humans and wildlife to certain chemicals in the environment will be greatly bolstered by an increased

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understanding of the xenobiotic's biological mechanism of action. With the advent of molecular toxicology, we are now able to classify complex mixtures of chemicals in the environment based on altered gene expression and hence can take advantage of this mechanistic information. Altered gene expression, whether assessed in a reporter assay or determined in a biological sample by RT-PCR, can indicate exposure and response to a wide array of chemical insults. Upon identification of exposure to a class of dangerous chemicals, i.e. dioxins or PCBs, the proper steps may be taken to ameliorate the situation. Also, if the sequence of events initiated by the chemical that results in toxic responses is better understood, we may be able to predict the consequences more accurately and provide effective clinical remedies. Therefore, the effective use of molecular biomarkers such as those discussed above may be important new tools in ecotoxicology and environmental epidemiology.

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Acknowledgements

Current work on alteration of gene expression by xenobiotics is supported by the National Institutes of Health (NIH DK49009 and NIH ES 07799). Previous support from the National Institute of Environmental Health Sciences and the National Toxicology Program is gratefully recognized.

References

ALAM, J. and COOK, J. L. 1990, Reporter genes: application to the study of mammalian gene transcription. *Analytical Biochemistry*, **188**, 245–254.

ASHELY, C. M., SIMPSON, M. G., HOLDICH, D. M. and BELL, D. R. 1996, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin is a potent toxin and induces cytochrome P450 in the crayfish. *Pacifastacus leniusculus*. *Aquatic Toxicology*, **35**, 157–169.

BALAGUER, P., JOYEUX, A., DENISON, M. S., VINCENT R., GILLESBY, B. E. and ZACHAREWSKI, T. 1996, Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using *in vitro* recombinant receptor-reporter gene assays. *Canadian Journal of Physiology and Pharmacology*, **74**, 216–222.

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W. W. and PRASHER, D. C. 1994, Green fluorescent protein as a marker for gene expression. *Science*, **263**, 802–805.

DE POMERAI, D. 1996, Heat shock proteins as biomarkers of pollution. *Human and Experimental Toxicology*, **15**, 279–285.

DICKERSON, R. L., HOOPER, M. J., GARD, N. W., COBB, G. P. and KENDALL, R. J. 1994, Toxicological foundations of ecological risk assessment: biomarker development and interpretation based on laboratory and wildlife species. *Environmental Health Perspectives*, **102**, 65–69.

FOSSI, M. C. 1994, Nondestructive biomarkers in ecotoxicology. *Environmental Health Perspectives*, **102**, 49–54.

GILLILAND, G. S., PERRIN, K. and BUNN, H. F. 1990, Competitive PCR for quantitation of mRNA. In *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds (San Diego, CA: Academic Press, Inc.), pp. 60–69.

GRANDJEAN, P., BROWN, S. S., REAYEY, P. and YOUNG, D. S. 1994, Biomarkers of chemical exposure: state of the art. *Clinical Chemistry*, **40**, 1360–1362.

KAIN, S. R. and GANGULY, S. 1996, Use of fusion genes in mammalian transfection. In *Current Protocols in Molecular Biology*, vol. 1, F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl, eds, (New York, NY: John Wiley & Sons, Inc.), pp. 9.6.1–9.6.12.

LINKS, J. M., KENSLER, T. W. and GROOPMAN, J. D. 1995, Biomarkers and mechanistic approaches in environmental epidemiology. *Annual Reviews of Public Health*, **16**, 83–103.

MACGREGOR, J. T., FARR, S., TUCKER, J. D., HEDDLE, J. A., TICE, R. R. and TURTLETAUB, K. W. 1995, Symposia overview: new molecular endpoints and methods for routine toxicity testing. *Fundamental and Applied Toxicology*, **26**, 156–173.

MATTES, W. B. 1997, The basics of the polymerase chain reaction. In *PCR Protocols in Molecular Toxicology*, J. P. Vanden Heuvel, ed. (Boca Raton, FL: CRC Press), pp. 1–40.

MCLACHLAN, J. A. 1993, Functional toxicology: a new approach to detect biologically active xenobiotics. *Environmental Health Perspectives*, **101**, 386–387.

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NRC 1989, *Biological Markers in Reproductive Toxicology* (Washington, DC: National Academy Press).

SEWALL, C. H., BELL, D. A., CLARK, G. C., TRITSCHER, A. M., TULLY, D. B., VANDEN HEUVEL, J. and LUCIER, G. W. 1995, Induced gene transcription: implications for biomarkers. *Clinical Chemistry* **41**, 1829–1834.

TODD, M. D., LEE, M. L., WILLIAMS, J. L., NALEZNY, J. M., GEE, P., BENJAMIN, M. B. and FARR, S. B. 1995, The CAT-Tox (L) Assay: a sensitive and specific measure of stress-induced transcription in transformed human liver cells. *Fundamental and Applied Toxicology*, **28**, 118–128.

VANDEN HEUVEL, J. P. 1997, Analysis of gene expression. In *PCR Protocols in Molecular Toxicology*, J. P. Vanden Heuvel, ed. (Boca Raton, FL: CRC Press), pp. 41–98.

VANDEN HEUVEL, J. P., CLARK, G. C., THOMPSON, C. L., MCCOY, Z., MILLER, C. R., LUCIER, G. W. and BELL, D. A. 1993, CYP1A1 mRNA levels as a human exposure biomarker: use of quantitative polymerase chain reaction to measure CYP1A1 expression in human peripheral blood lymphocytes. *Carcinogenesis*, **14**, 2003–2006.

VINCENT, R., GOEGAN, P., JOHNSON, G., BROOK, J. R., KUMARATHASAN, P., BOUTHILLER, L. and BURNETT, R. T. 1997, Regulation of promoter-CAT stress genes in HEPG2 cells by suspensions of particles from ambient air. *Fundamental and Applied Toxicology*, **39**, 18–32.

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