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Short Communication

QUANTITATIVE MEASUREMENT OF FATHEAD MINNOW VITELLOGENIN mRNA USING HYBRIDIZATION PROTECTION ASSAYS

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Abstract—We have developed a novel test system for the quantitative assessment of gene transcription. The procedure involves the use of chemiluminescent-labeled oligonucleotide probes in a hybridization protection assay (HPA) format. We have used this technology to measure changes in vitellogenin mRNA to demonstrate the impact of estrogen exposure in the juvenile fathead minnow (*Pimephales promelas*). Marked changes in mRNA expression were observed in response to intraperitoneal injection of 17β-estradiol demonstrating the utility of this technique for the identification and monitoring of toxic responses to xenobiotics.

Keywords—Hybridization protection assay Endocrine disruption Fathead minnow Vitellogenin Gene expression

INTRODUCTION

The potential problems of environmental pollution now attract widespread public concern. Moreover, increasing regulatory pressure is exposing a huge requirement for the reliable identification of environmental pollutants and their impact on human health and on wildlife. This in turn provides an impetus for the development of diagnostic tests that are capable of providing such information. One area to attract significant attention in recent years is the potential impact of endocrine-disrupting chemicals on the reproductive capability of various species, including humans [1,2]. A wide range of both natural and synthetic compounds has been implicated, including phytoestrogens, ethinyl estradiol, alkyl phenols, and polychlorinated biphenyls. Despite widespread demand for screening programs to aid the identification of such chemicals, much controversy remains as to the most appropriate methodology for this purpose [3; http://www.epa.gov/scipoly/oscpendo/history/finalrpt.htm]. A rational approach has been the search for suitable biomarkers that might be used as indices of potential disruption and so facilitate the establishment of a tiered testing system. The production of vitellogenin (VTG), a precursor to egg yolk protein, is used as an indicator of estrogen exposure in oviparous vertebrates. Under normal conditions, the production of VTG is induced by 17β-estradiol in the liver of female fish, but levels remain extremely low in male or juvenile fish. However, immunoassays of VTG [4,5] in various fish species have demonstrated inappropriate protein synthesis in male and immature fish exposed to estrogenic substances. A more direct approach is to monitor levels of VTG gene expression by measuring transcribed mRNA [6–8], though the limited use of mRNA measurement as an endpoint for toxicological investigation to date has reflected the complexity and expense of technology available.

Here we report the development of a chemiluminescent hybridization protection assay for the assessment of VTG gene activity induced by the exposure of animals or tissues to estrogens. In comparison with other methods such as northern blot analysis, RNAse protection assays, and RT-polymerase chain reaction, HPA is quantitative, sensitive, and easy to perform.

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MATERIALS AND METHODS

Test organisms

Mixed-sex juvenile fathead minnows were cultured at Water Research Centre (Medmenham, UK). Throughout each experiment, fish were maintained under flow-through conditions in water at 25 ± 2°C with a 16:8-h light:dark photoperiod. They were fed Tetramin® fish food (Tetra, Melle, Germany) daily at 4% of wet body weight.

Intraperitoneal injection

To characterize induction of VTG mRNA, mixed-sex juvenile fathead minnows were anesthetized in a solution of tricaine methanesulfonate (MS222) (1.0 g/L) and given intraperitoneal injections of 17β-estradiol (E_2) in 10% ethanol. The fish were injected with 10 μl/g of body weight to achieve a dose of 5 mg/kg. Control fish received injections of ethanol only. After injection, fish were held in 14-L all-glass aquaria filled with groundwater with gentle aeration, using an air diffuser stone. Temperature, pH, and dissolved oxygen concentration were measured at 24-h intervals throughout the experiment. At 1, 3, 5, and 7 d, five fish from each treatment were anesthetized as explained previously and then snap-frozen in liquid nitrogen. All samples were stored at −80°C until analyzed.

Preparation of total RNA

Total RNA was prepared from fish whole-body homogenate using TRI reagent (Sigma, Dorset, UK) using the protocol supplied by the manufacturer. The RNA samples were evaluated for purity and quantified by spectrophotometry (GeneQuant, Amersham Pharmacia Biotech, Cardiff, UK). Ribonucleic acid integrity was analyzed by electrophoresis, using

a 1% agarose gel prepared using sterile 1× TBE (Gibco BRL, Life Technologies, Paisley, Scotland). Samples of RNA were stored at −80°C prior to assay.

Design and synthesis of chemiluminescent oligonucleotide probes

Chemiluminescent oligonucleotide probes were prepared essentially as described by Nelson et al. [9]. A region of the fathead minnow (*Pimephales promelas*) vitellogenin gene (Genbank accession no. AF130354) was selected. A 23-base oligomer complementary to the section of the mRNA transcript (position = 3433–3456, 5′ CGG GCA AT#G ACA GCA AAA ACA GG 3′) was synthesized that contained a nonnucleotide, amine-terminated linker to which a chemiluminescent acridinium ester (AE) was attached (# indicates position of the nonnucleotide linker). The labeled oligonucleotide (AE probe) had a specific activity of approximately 2×10^6 relative light units (RLU) per picomole. In order to minimize any potential inhibition of hybridization caused by secondary structure of RNA transcripts, unlabeled oligonucleotides (30 mers), complementary to the flanking regions of the target sequence, were incorporated into the reaction [10].

Detection of chemiluminescence

Chemiluminescence was measured in a luminometer (Stratec Electronic, Birkenfeld, Germany) by automatic sequential injection of 200 μl HPA detection reagent 1 (0.032M hydrogen peroxide, 0.001M nitric acid) and 200 μl HPA detection reagent 2 (1.575M sodium hydroxide), followed by measurement of the light emission for 5 s.

Optimization of hybridization protection assays

A solution of AE probe in hybridization buffer (0.1 M lithium succinate, pH 5.0, 2 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol-bis [β-aminoethyl ether]-*N,N,N′,N′*, tetraacetic acid, 10% [w/v] lithium lauryl sulfate), which had been incubated previously (60°C for 30 min) with and without complementary target, was admixed with selection reagent (190 mM sodium tetraborate, 6.4% Triton X-102 pH 7.5 from Sigma) and incubated at 60°C over a range of time points. Immediately after removal from the water bath, tubes were placed in ice/water slurry for 2 min, followed by a further 2 min at room temperature before chemiluminescence was measured. Data were plotted as the log percentage of initial chemiluminescence (at time zero) versus time, and hydrolysis rates were calculated from the slope using standard regression analysis.

Measurement of VTG mRNA

Lyophilized AE probe was reconstituted in 6 ml of hybridization buffer. Samples of total RNA, prepared from fish whole-body homogenate, were measured in duplicate. Each measurement was performed in 12 × 75-mm assay tubes into which either 50 μl of total RNA or synthetic target oligonucleotide at 0, 10, 40, 400, and 800 fmol/ml were added followed by 50 μl of VTG AE probe (0.1 pmol). The samples were vortexed, covered, and placed in a water bath at 60°C for 30 min. Selection reagent (300 μl) was added, and the samples were incubated for a further 15 min at 60°C. The reaction was stopped by placing in an ice/water slurry for 2 min, followed by a further 2 min at room temperature before chemiluminescence was measured.

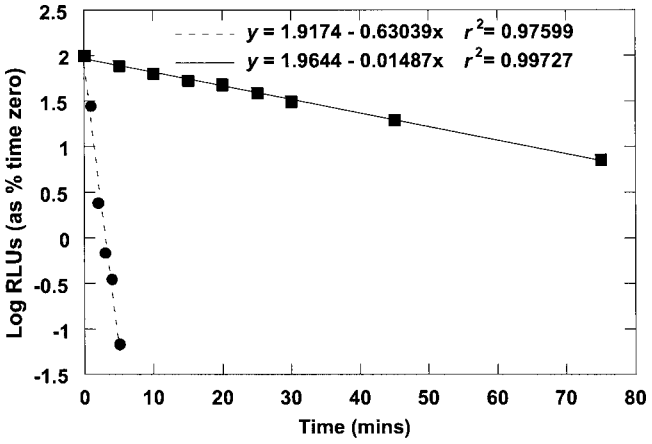

Fig. 1. Acridinium ester hydrolysis of hybridized (■) and unhybridized (●) probe is shown as loss of chemiluminescence activity over time. Residual chemiluminescence activity, expressed as a percentage of the activity of unhydrolyzed label, is plotted relative to the time of exposure to hydrolyzing conditions. The equations generated from regression analyses for the hybridized and unhybridized labeled probe are given along with the r^2 values for each fit; RLU = relative light units.

Assay precision

The fathead minnow VTG gene was cloned, and RNA was prepared using the Promega RiboMAX (T3) system (Madison, WI, USA) according to the manufacturer's instruction. The concentration of the RNA measured by spectrophotometry was 10 μg/μl. This solution was diluted to 1:5,000, 1:10,000, 1:100,000 and heated to 60°C for 5 min, and a HPA assay was performed as described previously. Concentrations were determined by interpolation of the calibration curve. Intra-assay precision was calculated from the measurement of six replicate samples. Inter-assay precision was calculated from the mean of duplicate sample measurements performed over six assays.

RESULTS AND DISCUSSION

The HPA technology is based on chemiluminescent technology that has been described previously [9,11]. Here we describe the application of this technology to quantitatively measuring the mRNA levels of specific transcript in total RNA. The HPA has been applied to monitoring vitellogenin gene activity in response to intraperitoneal injection of E_2 in juvenile fathead minnow.

The AE molecule reacts rapidly with hydrogen peroxide under alkaline conditions to produce light at 430 nm. The position of the chemiluminescent label in the oligonucleotide is chosen such that the duplex affords maximum protection of the acridinium salt from contrived hydrolytic degradation, thus preserving its chemiluminescent properties [11]. The HPA consists of two steps: The AE probe is hybridized to its target, and then addition of the alkaline reagent causes preferential hydrolysis of unhybridized probe. Chemical hydrolysis of the ester bond of the AE molecule renders the AE permanently nonchemiluminescent. Figure 1 shows the differential rate at which probe hybridized to its specific complementary target and unhybridized probe was hydrolyzed at 60°C over a range of time points. In the absence of target, the probe is degraded rapidly. This is in marked contrast to probe hybridized to its complementary target, which is protected from hydrolysis. This differential hydrolysis is responsible for the very low background chemiluminescence in the assay. Following mea-

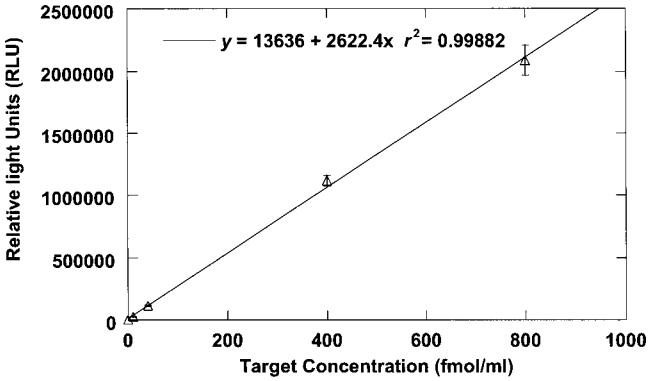

Fig. 2. Standard curve for the vitellogenin (VTG) mRNA hybridization protection assay (HPA). The curve represents the mean of six assays, and error bars are ± 1 standard deviation. The mean assay response at zero dose was 2,000 relative light units (RLU).

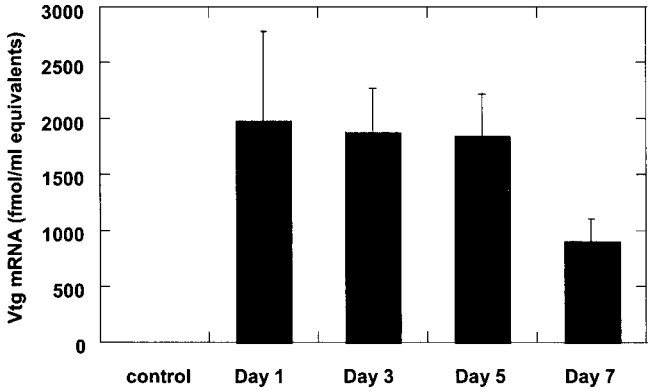

Fig. 3. Intraperitoneal exposure of fathead minnow to 17β-estradiol (E_2) at a dose of 5 mg/kg body weight. The E_2 was administered on day 0, and fish (n = 5) were sampled on days 1, 3, 5, and 7. Data points represent mean values ± standard error of mean (n = 5); VTG = vitellogenin.

surement of chemiluminescence at each time point, an optimum time was selected that gave the greatest range of chemiluminescence intensity between hybridized and unhybridized states. For the VTG HPA assay, a hydrolysis time of 15 min was used in all further experiments. Typically, the signal/noise ratio under these conditions was 1,000:1.

A typical calibration curve for the assay is shown in Figure 2 illustrating the linearity of chemiluminescent response between 0 and 800 fmol/ml of oligonucleotide. The sensitivity of detection of the assay was 10^{-16} mol (0.1 femtomol). To measure the intra-assay and inter-assay variation, replicate measurements were carried out on in vitro transcribed RNA (Table 1). The largest variation, 7.1% at a dose of 15 fmol/ml, demonstrates the highly reproducible performance.

Duplicate mRNA measurements were made of total RNA samples from each of the juvenile fathead minnows exposed to E_2 via IP injection. Figure 3 shows the VTG mRNA response over the 7-d period. Specific mRNA was undetectable in control fish but reached a maximum of approximately 2,000 fmol/ml after a 1-d exposure to E_2. The rapidity of the response to IP exposure can be seen from the appearance of significant gene activity within a day following exposure. Vitellogenin mRNA decreased toward baseline levels over the 7-d period monitored. Our studies demonstrate that the HPA assay can be used to monitor the substantial changes in VTG gene activity that occur in response to exposure. The excellent precision of the assay would suggest that the variation seen reflects individual differences in response to E_2.

This analytical system offers several unique advantages to other techniques used to measure gene expression. The measurement of target in a single-tube homogeneous assay allows detection of the gene product without a reverse transcription or an amplification step and provides a direct quantitation of the amount of target present. This also precludes the contamination problems that can occur with polymerase chain reaction amplification. The hybridization protection assay eliminates the manipulation steps required by northern and RNase protection assays and avoids the necessity of radiolabeled probes. Perhaps the most important advantage of the technology is its flexibility. The measurement of specific mRNA transcripts by HPA is a method easily adapted to quantitate any response that involves a change in gene expression, either in vivo or in vitro, and it can be applied to many areas of toxicological investigation. The nature of differential hydrolysis makes the HPA reaction extremely specific, as even a single mismatch in the target sequence can lead to complete loss of the chemiluminescent signal [9]. The assay is also easy to perform and rapid compared to other technologies.

A need is increasing for information on the toxicological properties and the potential impact on living organisms of the many chemicals in widespread use as well as on chemicals under development. Traditional investigations based on life parameters such as growth and mortality are inappropriate for monitoring subtle responses or for screening large numbers of compounds. While recognizing a continuing requirement for conventional bioassays, regulatory authorities are actively seeking simple analytical methods that can facilitate the introduction of a tiered testing system [3]. High sensitivity, specificity, and ease of use combine to make this technology suitable for widespread application in the identification of toxic compounds and the monitoring of their impact on the biosphere. We believe that monitoring genetic responses in this manner will become routine in the investigation of the toxicological properties of compounds and their impact on environmental health.

Table 1. Precision of measurement of the hybridization protection assay for vitellogenin (VTG) mRNA

VTG mRNA (fmol/ml)	Intra-assay coefficient of vairation(CV)[a]	Inter-assay coefficient of variation (% CV)
15	3.3	7.1
155	2.9	4.3
295	2.2	4.0

[a] CV = coefficient of variation.

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