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Development of a Green Fluorescent Protein-Based Cell Bioassay for the Rapid and Inexpensive Detection and Characterization of Ah Receptor Agonists

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The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates the toxic and biological effects of a variety of chemicals. Although halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs, respectively) represent the highest affinity and most toxic ligands, recent studies have demonstrated that the AhR can be activated by chemicals with structures distinctly different from HAHs/PAHs. In order to identify and characterize novel AhR ligands, we developed a rapid and inexpensive high-throughput screening bioassay based on the ability of AhR agonists to induce an HAH/PAH-responsive, enhanced green fluorescent protein (EGFP) reporter gene in a stably transfected mouse hepatoma (Hepa1c1c7) cell line. EGFP induction in the resulting recombinant cell line, H1G1.1c3, is sensitive (with a minimal 1-pM detection limit for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most potent AhR ligand), and it responds to HAHs and PAHs in a time-, dose-, and chemical-specific manner. Application of this bioassay was demonstrated by the rapid characterization of the relative inducing potency of a series of previously uncharacterized dioxin surrogates. This bioassay system has numerous advantages over currently available AhR-based bioassays including increased rapidity and ease of use, low reagent cost, and application for high-throughput screening.

Key Words: **dioxin, Ah receptor, green fluorescent protein, TCDD, HAH, GFP.**

The aryl hydrocarbon receptor (AhR) is a soluble, ligand-dependent transcription factor that regulates the induction of gene expression by virtue of its ability to bind ligands, translocate into the nucleus, and interact with specific regulatory DNA elements (dioxin-responsive elements or DREs) (Denison *et al.*, 1998a; Whitlock, 1999). Although ligands for AhR include a diverse range of structurally dissimilar chemicals (Denison *et al.*, 1998c; Safe, 1990), ligands with the highest affinity include specific halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs, respectively), the majority of which are widespread environmental contaminants. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent HAH and AhR ligand, not only results in activation of AhR and the induction of expression of a battery of genes, but also results in a variety of toxic and biological effects at extremely low concentrations. The adverse effects of TCDD and closely related HAHs are species- and tissue-specific and include lethality, tumor promotion, teratogenicity, immune suppression, and alterations in endocrine homeostasis (reviewed in Devito and Birnbaum, 1994). The role of the AhR in mediating the effects of TCDD has been recently confirmed using AhR knockout mice (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997).

The widespread nature of TCDD and related chemicals and their ability to biomagnify in the food chain and produce adverse effects in humans and animals (Devito and Birnbaum, 1994; Giesy *et al.*, 1994a,b; Mocarelli *et al.*, 2000) has generated considerable concern worldwide. Accordingly, numerous analytical techniques have been developed for the detection and quantification of these chemicals in environmental, biological, and food samples in order to assess their formation and sources, fate, transport, exposure, and body burdens. Quantitative extraction procedures coupled with high-resolution gas chromatography/mass spectrometry (HR GC/MS) have been developed to allow accurate detection of HAHs at the part-per-quadrillion level (Clement, 1991; Stephens *et al.*, 1992). Although these instrumental analysis procedures provide an accurate measurement of these chemicals in sample extracts and are the "gold standard" for HAH analysis, the procedures are extremely costly, time consuming, have relatively low sample throughput, and require expensive and sophisticated equipment. These aspects are problematic, especially when the experimental design or monitoring program requires large numbers of samples to be screened. Although analytical chemical quantification of HAHs does not provide any information as to the toxic potency, the combination of these data with toxic equivalency factors (TEFs) for HAHs provides an avenue on which to estimate the potency of a complex HAH mixture (Safe, 1990; Van den Berg *et al.*, 1998). In this method, the total dioxin-like activity is estimated by multiplying the con-

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centration of a given HAH by its TEF, which is a factor expressing AhR-dependent toxicity relative to TCDD. The sum of the resulting values for all HAHs represents the toxic equivalency (TEQ) of the mixture. However, this approach has numerous limitations, including its inability to assess chemical interactions (synergism or antagonism) that can increase or decrease the potency of a given mixture. In addition, the established HRGC/MS procedures are specifically targeted to HAHs and do not allow for the identification of other novel chemicals and chemical classes that could activate the AhR and thus alter the toxic potency of a complex chemical mixture. Thus, the TEF approach could readily result in an underestimation or an overestimation of the potency of a complex mixture.

Given the specificity of the AhR for the majority of HAHs of concern, most currently developed bioassay systems take advantage of one or more aspects of the AhR-dependent mechanism of action (El-fouly *et al.*, 1994; Garrison *et al.*, 1996; Murk *et al.*, 1996; Postlind *et al.*, 1993; Richter *et al.*, 1997; Sanderson *et al.*, 1996; Ziccardi *et al.*, 2000). These bioassay systems are gaining widespread use as rapid, low-cost, and sensitive screening methods for the detection and relative quantification of dioxin-like HAHs and PAHs in sample extracts. In addition, these systems have been utilized to identify and characterize novel chemicals and classes of chemicals that can activate the AhR (Denison *et al.*, 1998c; Heath-Pagliuso *et al.*, 1998; Phelan *et al.*, 1998; Seidel *et al.*, 2000). The most sensitive bioassay systems that have been developed to date are with recombinant cell lines that contain a stably transfected dioxin (AhR)-responsive firefly luciferase gene that we and other investigators have developed (Garrison *et al.*, 1996; Murk *et al.*, 1996; Postlind *et al.*, 1993; Richter *et al.*, 1997; Sanderson *et al.*, 1996; Ziccardi *et al.*, 2000). Treatment of these cells with TCDD and related HAHs and PAHs, as well as other AhR ligands, results in induction of reporter gene expression in a time-, dose-, AhR-, and chemical-specific manner. The level of reporter gene expression correlates with the total concentration of the TCDD-like AhR agonists in the sample. Although the firefly luciferase reporter gene contributes to the high degree of sensitivity of the assay, it has limitations with respect to our need for a rapid and inexpensive bioassay for high-throughput screening analysis. Here we describe the development, optimization, and characterization of a novel screening system that responds to AhR agonists with the induction of enhanced green fluorescent protein (EGFP). This new bioassay not only has the same sensitivity and chemical specificity as our previously described, luciferase-based cell bioassays, but it is easier, more rapid, and less expensive, and reporter gene activity can be measured in "real time."

MATERIALS AND METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was a gift from Dr. Steven Safe (Texas A&M University); 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PCDF), 3,3′,4,4′,5-pentachlorobiphenyl (3,3′,4,4′,5-PCB), 3,3′,4,4′-tetrachlorobiphenyl (3,3′,4,4′-PCB), 1,2,4,7,8-pentachlorodibenzo-*p*-dioxin (1,2,4,7,8-PCDD), benzo(*a*)pyrene (BAP), benz(*a*)anthracene (BAA), benzo(*k*)fluoranthene (BKF), and dibenz(*a*,*h*)anthracene (DB(*a*,*h*)A) were purchased from Accustandards (New Haven, CT). Many of these chemicals are extremely toxic and/or carcinogenic, and they were handled and disposed of as previously described (Garrison *et al.*, 1996). Synthesis of the dioxin surrogates was previously described by Sanborn *et al.* (1998). [γ^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL), fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA), Geneticin (G418) and all tissue culture media from Gibco/BRL (Grand Island, NY), and DNA modification enzymes from New England Biolabs (Beverly, MA).

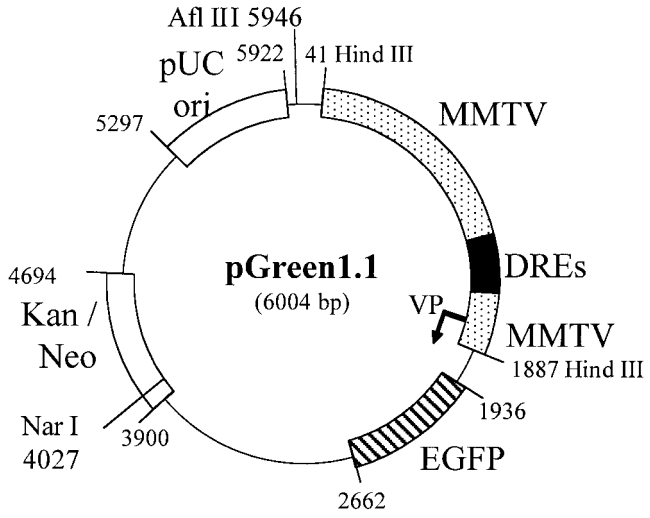


FIG. 1. Structure of the dioxin-responsive EGFP reporter plasmid, pGreen1.1. This vector contains the 1846 base-pair (bp) Hind III fragment from the plasmid pGudLuc1.1 inserted into the Hind III site of the reporter plasmid pEGFP-1 (Clontech). This fragment contains the 480-bp dioxin-responsive domain with 4 DREs isolated from the upstream region of the mouse CYP1A1 gene inserted into the mouse mammary tumor virus (MMTV) long terminal repeat. The 4 DREs confer TCDD-responsiveness upon the MMTV viral promoter (VP) and EGFP reporter genes. Also contained in pEGFP1.1 is the pUC origin of replication and a Kanamycin/Neomycin resistance gene for selection.

Construction of GFP expression vector. The expression vector pGreen1.1 (Fig. 1) was created by excising the 1846 base-pair (bp) Hind III fragment from the plasmid pGudLuc1.1 (Garrison *et al.*, 1996). This fragment contains the 480-bp dioxin-responsive domain from the mouse CYP1A1 gene inserted upstream of the mouse mammary tumor virus (MMTV) promoter and it confers dioxin responsiveness upon the MMTV promoter and adjacent reporter gene. This fragment was inserted into the Hind III site immediately upstream of the EGFP reporter gene in the plasmid pEGFP-1 (Clontech, Palo Alto, CA).

Cell culture and stable transfections. The mouse hepatoma (Hepa1c1c7) cell line was maintained in nonselective media (αMEM containing 10% fetal bovine serum). Plates of cells (approximately 80% confluent) were transfected with the construct pGreen1.1 (20 μg) using polybrene as previously described (Garrison *et al.*, 1996). After growth in nonselective media for 24 h, cells were split 1 to 10 and replated into selective media (nonselective media containing 968 mg/l of the antibiotic G418). After growth in selective media for 4 weeks, resistant clones were isolated and screened for the induction of EGFP expression by treatment of cells with 1 nM TCDD for 24 h. Clones exhibiting the highest ratio of inducible to constitutive EGFP expression were further characterized.

Chemical treatment and measurement of EGFP. In initial experiments, H1G1.1c3 cells were plated into 6-well culture plates and treated with DMSO

(1% maximum final concentration), or TCDD (1 nM in DMSO) for 24 h at 37°C. Cells were harvested by scraping into lysis buffer (50 mM NaH_2PO_4, 10 mM Tris–HCl pH 8, 200 mM NaCl), and the cells were lysed by repeated passage through a 27-gauge needle. Samples were centrifuged and the fluorescence of an aliquot of the supernatant was determined in a Perkin Elmer LS 50B fluorometer using excitation and emission wavelengths of 460 nm and 510 nm, respectively. For microtiter plate analysis of EGFP in intact cells, cells were plated into black, clear-bottomed 96-well tissue culture dishes (Corning, San Mateo, CA) at 75,000 cells per well and allowed to attach for 24 h. Selective media was then replaced with 100 μl of nonselective media containing the test chemical or DMSO (1% final solvent concentration). EGFP levels were measured in the intact cells (without removal of media) at the indicated time points, using a Fluostar microtiter plate fluorometer (Phoenix Research Products) with an excitation wavelength of 485 nm (25-nm bandwidth) and an emission wavelength of 515 nm (10-nm bandwidth). In order to normalize results between experiments, the instrument fluorescence gain setting was adjusted so that the level of EGFP induction by 1 nM TCDD produced a relative fluorescence of 9000 relative fluorescence units (RFUs). Samples were run in triplicate, and the fluorescent activity present in wells containing media only were subtracted from the fluorescent activity in all samples.

Fluorescence microscopy. H1G1.1c3 cells were grown on 25-mm round cover slips for 24 h and then treated with DMSO or 1 nM TCDD for 48 h. To photograph the cells, the media was replaced with phosphate-buffered saline, and cell fluorescence was visualized using an Olympus BH2 fluorescence microscope with a 490-nm excitation filter and a 535-nm emission filter.

Measurement of luciferase activity in recombinant mouse hepatoma (H1L1.1c2) cells. H1L1.1c2 cells, which contain a stably integrated, DRE-driven firefly luciferase reporter gene plasmid whose transcriptional activation occurs in a ligand- and AhR-dependent manner, were maintained as previously described (Garrison *et al.*, 1996). These cells, grown in 24-well microplates, were incubated with DMSO (10 μl/ml), the indicated concentration of TCDD or surrogate in DMSO for 4 h at 37°C. After incubation, luciferase activity in cells in each well were determined, as we have previously described in detail (Denison *et al.*, 1998b). Luciferase activity was normalized to sample protein concentration using fluorescamine (Ziccardi *et al.*, 2000), with bovine serum albumin as the standard.

Animals and preparation of cytosol. Male Hartley guinea pigs (250–300 g), obtained from Charles River breeding laboratories (Wilmington, DE), were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water. Hepatic cytosol was prepared in HEDG buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol) as described (Denison *et al.*, 1986) and stored at −80°C until use. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Synthetic oligonucleotides and gel retardation analysis. A complementary pair of synthetic oligonucleotides containing the sequence 5′-GATCTG-GCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGA-AGAGCCA-3′ (corresponding to the AhR binding site of DRE3 and designated as the DRE oligonucleotide) was synthesized, purified, annealed, and radiolabeled with [γ^{32}P]ATP as described (Denison *et al.*, 1988). Gel retardation analysis of cytosolic AhR complexes transformed *in vitro* with TCDD (20 nM) or the indicated compound was carried out as previously described (Denison *et al.*, 1998b) and protein–DNA complexes were visualized by autoradiography. The amount of [^{32}P]-labeled DRE present in the induced protein–DNA complex was determined using a Molecular Dynamics phosphorimager.

RESULTS

Generation of a Stably Transfected Cell Line

We have previously used mouse hepatoma (Hepa1c1c7) cells to generate a stably transfected cell line (H1L1.1c2) containing an AhR-responsive, luciferase-based reporter gene to detect and quantify HAHs, PAHs, and other AhR agonists (Denison *et al.*, 1998b; Heath-Pagliuso *et al.*, 1998; Phelan *et al.*, 1998; Seidel *et al.*, 2000; Ziccardi *et al.*, 2000). The Hepa1c1c7 cells provide an excellent model cell line because of their high degree of responsiveness to TCDD and related chemicals. This is due in large part to the unusually high concentration of AhR complexes in these cells (Holmes and Pollenz, 1997). To generate a stably transfected cell line containing an AhR-responsive EGFP reporter, we utilized the approach we had used to create the stably transfected H1L1.1c2 cells. The enhanced green fluorescent protein (EGFP) reporter gene is a modified form of the green fluorescent protein isolated from *Aequoria victoria* that has been optimized for mammalian expression (Cormack *et al.*, 1996; Inouye and Tsuji, 1994; Prasher *et al.*, 1992). Prior to stable transfection, we confirmed the TCDD responsiveness of the plasmid pGreen1.1 (Fig. 1) in transient transfection experiments with hepa1c1c7 cells and found that TCDD could induce expression of EGFP in these cells (data not shown). Hepa1c1c7 cells were then stably transfected with pGreen1.1, and the induction of EGFP by TCDD in the isolated clones was determined. Of 10 initial cell clones tested, only 2 (clones 2 and 3) responded to TCDD with a significant induction of EGFP activity (Fig. 2). Given the greater degree of responsiveness of clone 3, this clonal cell line was further characterized and is hereafter referred to as the H1G1.1c3 cell line.

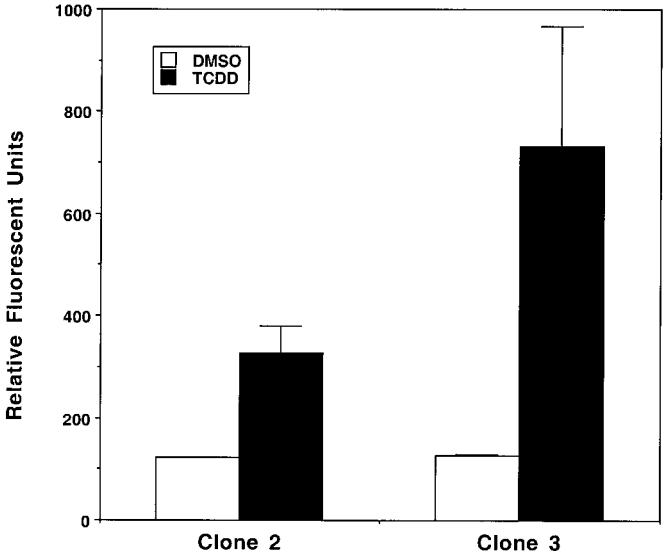


FIG. 2. Induction of EGFP expression by TCDD in 2 stably transfected cell clones. Neomycin-resistant colonies of Hepa1c1c7 cells that had been stably transfected with pGreen1.1 were treated with DMSO or 10^{-9} M TCDD for 24 h at 37°C, lysed, and the fluorescence of the lysates measured as described in Materials and Methods. Values represent the mean ± SD of 3 determinations. *Values were significantly greater than that of the DMSO control ($p < 0.05$, Student′s *t*-test).

A major advantage associated with EGFP as a reporter gene is its ability to be measured in intact cells. To visually examine

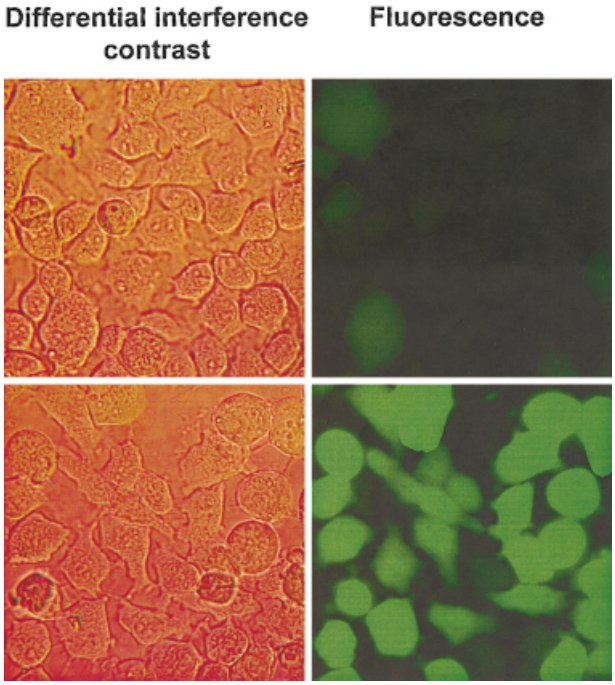


FIG. 3. Microscopy of EGFP1.1 cells incubated in the presence of DMSO or TCDD. H1G1.1c3 cells were grown on 25-mm cover slips until 80% confluent and then treated with DMSO or 10^{-9} M TCDD for 48 h at 33°C. Cells were visualized under differential interference contrast microscopy, or under fluorescence microscopy as described in Materials and Methods.

induction of EGFP expression, H1G1.1c3 cells were treated with DMSO or 1 nM TCDD and examined by fluorescence microscopy (Fig. 3). Although there was considerable variation in fluorescence in individual control and treated cells under these conditions, TCDD caused a dramatic increase in overall fluorescence. The low level of fluorescence in untreated H1G1.1c3 cells is due to constitutive expression of the EGFP reporter gene and not fluorescence of endogenous cellular components, since no fluorescence is observed in untransfected Hepa1c1c7 cells under these same conditions (data not shown).

Optimization of the H1G1.1c3 Cell Bioassay in Microtiter Plate Format

The ability to directly detect EGFP in intact H1G1.1c3 cells suggested that these cells could be used to further develop the bioassay for use in a 96-well microtiter plate format. A microtiter plate EGFP bioassay would have significant advantages over our recently developed luciferase microtiter plate bioassay given the lack of a lysis step or need for expensive reagent addition in order to measure reporter activity. These 2 aspects combined with the ability to read the fluorescence in an entire 96-well microtiter plate in less than 1 min would save time, reduce costs, and allow the bioassay to be adapted for high-throughput analysis. In initial experiments, we determined the optimal type and color of microtiter plate to be used in these assays. The results of these studies revealed that the greatest ratio of TCDD-induced expression over control and background fluorescence was attained when cell fluorescence was read from the bottom of a black, 96-well, clear-bottomed microtiter plate (data not shown). Reading fluorescence from the bottom of the plate also eliminated the necessity of removing the cell culture media from the well, increasing the speed and ease of sample analysis. Since the presence or absence of media in the sample wells had no effect on fluorescence (unpublished results), media were left in the wells, both for ease of analysis and to maintain cell sterility and viability during the reading of the plate.

In previous studies with the luciferase bioassay system, we have observed microtiter plate edge effects in white, clear-bottomed plates, wherein the activity of samples in the wells on the outer edge of the plate (rows A and H, and columns 1 and 12) was decreased compared to that of samples in the inner wells (data not shown). Since this phenomenon can result in an underestimation of actual reporter gene activity, we examined basal and 1 nM TCDD-induced activity in wells of black, clear-bottomed plates containing identical numbers of cells (data not shown). Although comparison of basal fluorescence revealed less than a 4% variation between any 2 wells in the plate, variation between wells in the induced plate were upwards of 10–15%. We also observed a slight downward trend in fluorescence across the plate containing the uninduced cells (from rows A to H) with no apparent edge effect. In contrast, significant edge effects were observed in wells containing induced EGFP activity, presumably due to the higher activity, with some reduction in activity observed in rows A and H; induced EGFP activity in column 12 of all rows was consistently lower than that in all other wells in the same row. Although there is some variation between individual wells, we still utilize all wells on a plate for high-throughput screening analysis when the specific endpoint is simply to identify positive inducers. However, when accurate quantitative assessment of the relative inducing potency of a given chemical(s) is desired, these wells should not be used, and all comparisons are based on EC_{50} values estimated from dose response curves rather then simply comparison to the maximal level of induction. The reproducibility of the assay was then examined by comparing the level of EGFP induction by TCDD (1 nM) in 33 wells of cells in 3 different plates run on 3 successive days. The similarity of the results revealed a high degree of reproducibility between runs (the induction values expressed as a mean $\pm$ SD for these analyses were 5276 $\pm$ 308, 4404 $\pm$ 135, and 4199 $\pm$ 236).

We next examined the basal and TCDD-inducible level of EGFP fluorescence from cells in the microtiter plate. H1G1.1c3 cells were incubated with DMSO or TCDD (1 nM) for 24 h and the EGFP expression determined (Table 1). In order to ensure a high signal-to-background ratio and to normalize results between experiments, the fluorometer gain setting was adjusted so that maximal induction resulted in 9000 RFUs. TCDD treatment resulted in a 7-fold induction of

TABLE 1
Comparison of the Green Fluorescent Emission from Control and TCDD-Treated H1G1.1c3 and Untreated Hepa1c1c7 Cells

Treatment	Relative fluorescence	Fold induction[b]	Corrected fluorescence[c]	Fold induction
H1G1.1c3				
DMSO	1311 ± 19[a]	–	497 ± 19	–
TCDD	9158 ± 138	7	8345 ± 138	17
Hepa1c1c7				
DMSO	818 ± 28	–	4 ± 28	–
TCDD	798 ± 15	0	−16 ± 15	0
Media alone				
NT	814 ± 16	–	0 ± 15	–

Note. NT, not treated.

[a]Values represent the mean ± standard deviation of 3 replicate analyses.

[b]Values represent the fold induction of fluorescence over that of the DMSO sample.

[c]Values represent the fluorescence activity after subtraction of that present in the media-alone sample.

fluorescence, while no change in fluorescence was observed in untransfected hepa1c1c7 cells. These data indicate that the increase in fluorescence is due to the induction of EGFP expression and not to a change in fluorescence of endogenous cellular component(s) in the cells themselves. In addition, these results indicate that approximately 60% of the background fluorescence comes from the medium. Subtraction of this background fluorescence (found with culture media only) from all samples revealed that TCDD treatment of H1G1.1c3 cells actually results in a 17-fold induction of EGFP expression (Table 1). In all subsequent experiments, the background fluorescence from wells containing culture medium only is subtracted from all results to provide an accurate assessment of EGFP activity.

The EGFP reporter gene used in our experiments is a variant of GFP that has been optimized for mammalian expression (Cormack *et al.*, 1996). Previous studies have reported that EGFP formation is temperature sensitive, with transiently transfected cells grown at 30–33°C exhibiting higher fluorescence than those grown at 37°C (Ogawa *et al.*, 1995; Pines, 1995). In order to determine whether a similar temperature dependence is observed with EGFP and to examine the time course of induction, we examined the level of TCDD-inducible EGFP expression in H1G1.1c3 cells grown at 33°C and 37°C. After 18 h of treatment, induction of EGFP activity in cells grown at 33°C was significantly higher than in cells grown at 37°C (Fig. 4), which is presumably a result of low rates of EGFP formation at 37°C; no significant difference in the fluorescence was observed with the control cells. For the remainder of the experiments, cells were maintained at 37°C and incubated with test chemicals at 33°C to maximize EGFP expression and inducible fluorescence. Because EGFP can be measured in intact cells, the entire time course was carried out in 6 wells of a 96-well plate at each temperature. After each reading, the plates of cells were returned to the incubator for rereading at subsequent time points. The ability to remeasure cell fluorescence at various time points is a significant advantage over previously described bioassays by permitting measurement of induction in "real time."

Characterization of the Responsiveness of H1G1.1c3 Cells

One major application of this new cell bioassay is the detection and characterization of new AhR agonists. Accordingly, for this cell line to be useful it should be sensitive and have an acceptable dynamic concentration range of response. Dose-response relationship experiments for induction of EGFP by TCDD in the H1G1.1c3 cells revealed a minimal detection limit (MDL) of ~1 pM, an EC_{50} of 18 pM, and maximal induction at ~300 pM (Fig. 5). The stability of the EGFP reporter ($T_{1/2}$ = 22 h) and the ability to remeasure EGFP activity in the same cells over time allowed analysis of the dose-response relationship over time (Fig. 6). As expected, the dose-response characterizations for EGFP induction (i.e., the MDL, EC50, and the maximal induction concentration) were consistent over time, with the 24-h time taken as the standard for analysis. The lack of deviation of the dose curves over time also indicates a consistency in the induction pathway, without other factors affecting its induction over time as is the case in our H1L1.1c2 cells, in which the reporter gene becomes rapidly downregulated after 4 h (Garrison *et al.*, 1996). Comparison of the responsiveness and sensitivity of the H1G1.1c3 cell

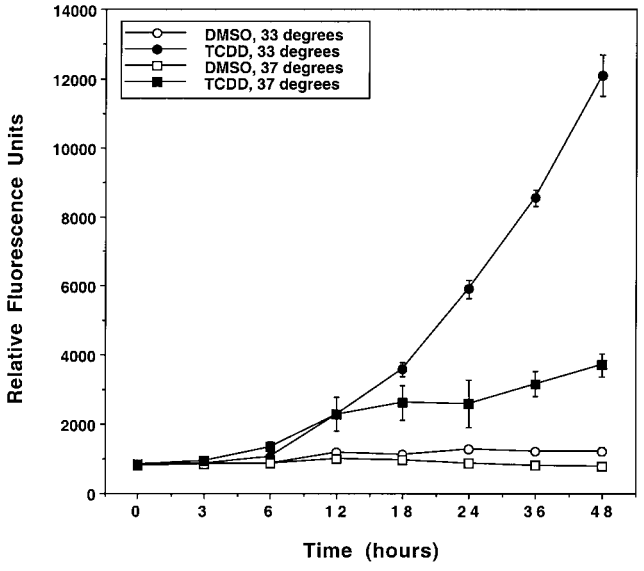


FIG. 4. Time course of induction of EGFP expression by TCDD in cells grown at different temperatures. H1G1.1c3 cells were grown in 96-well tissue-culture dishes as described in Materials and Methods. After treatment with 10^{-9} M TCDD or DMSO, the cells were incubated at 33°C and 37°C for the indicated time periods. Values represent the mean ± SD of 3 determinations. At both temperatures, TCDD-induction of EGFP fluorescence was significantly greater than the DMSO control ($p < 0.05$, Student's *t*-test) at times equal to or later than 6 h.

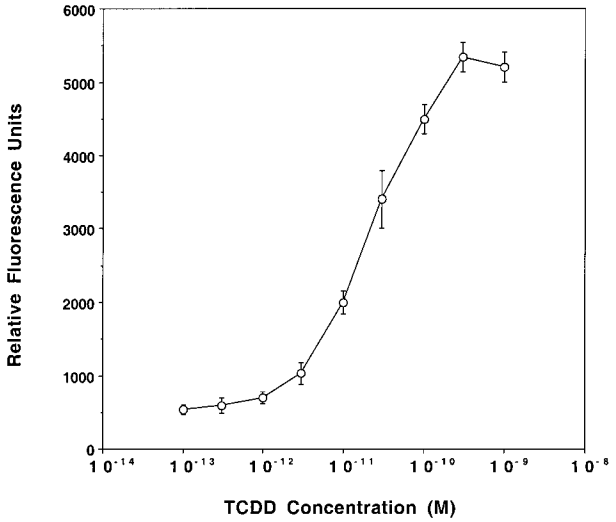


FIG. 5. TCDD dose-response relationship for induction of EGFP. H1G1.1c3 cells were treated with the indicated concentrations of TCDD for 24 h at 33°C and EGFP fluorescence was measured without the removal of the growth medium, as described in Materials and Methods. Values represent the mean ± SD of 3 determinations. Induction of fluorescence was significantly greater ($p < 0.05$, Student′s *t*-test) than control at 10^{-12} M TCDD.

bioassay to that of the other previously described AhR bioassays is presented in Table 2. The EC_{50} and MDL for the H1G1.1c3 cell line were very comparable to that of previously described luciferase cell bioassays. It should be noted that although the MDL for the H1G1.1c3 cells is the same as that for several other cell bioassays (1×10^{-12} M), the MDL expressed as pg/well is significantly lower that of most other bioassays (Table 2). This lower detection limit results from the smaller volumes used in the H1G1.1c3 cell microtiter plate bioassay protocol compared to those used in the other bioassays. Thus, in the microtiter plate format, the H1G1.1c3 cells are currently one of the most sensitive bioassay systems for detection of TCDD and related AhR agonists.

Our previous experiments demonstrated an AhR-dependent induction of reporter gene activity by TCDD and related agonists (Denison *et al.*, 1998b; Heath-Pagliuso *et al.*, 1998; Phelan *et al.*, 1998; Seidel *et al.*, 2000). Given the similarity in design of our pGreen1.1 plasmid to that of the dioxin-responsive, luciferase-based pGudLuc1.1 used in our H1L1.1c2 cell line, the induction of EGFP expression was expected to be AhR-dependent. To confirm this and to establish the relative potency of AhR ligands, we examined the ability of selected HAHs and PAHs to induce EGFP expression in H1G1.1c3 cells. The relative rank order potency for HAHs in these cells (Fig. 7A) was comparable to our previous results with the luciferase responsive H1L1.1c2 cells (Garrison *et al.*, 1996), and they correlate well with the rank order of potency of these chemicals to bind to AhR (Safe, 1990). Our results for the PAHs (Fig. 7B) demonstrate the greater induction potency of DB(ah)A and BKF as compared to BAA and BAP, and are consistent with our previous studies using PAHs (Ziccardi *et al.*, 2000). However the relative potency of BKF in the H1G1.1c3 cells was 10-fold lower as compared to our previous results using H1L1.1c2 DRE-luciferase cells (Ziccardi *et al.*, 2000). The decreased potency in H1G1.1c3 cells may be due to metabolism of BKF and reduction in potency during the 24-h treatment period as compared to the 4-h treatment period in H1L1.1c2 cells where minimal metabolism would occur. Overall, these results demonstrated the utility of this cell line as a bioassay system for the detection of chemicals that can activate the AhR signal transduction pathway.

Application of the H1G1.1c3 Cells to Determine the Relative Potency of TCDD Surrogate Standards

In previous studies, we reported the synthesis of a series of TCDD haptens and surrogate standards for the development of TCDD immunoassays (Sanborn *et al.*, 1998). These materials have several uses. Several of the compounds have functional groups allowing them to be coupled to proteins as reporter-group groups. Some of these haptens improved the sensitivity of some immunoassays because they bound to the antibody with lower affinity than the target analyte (TCDD), resulting in improved competition and improved sensitivity of the assay. The other use for these surrogates was in that many were detected by the antibody at levels similar to TCDD. This allowed the surrogate to be used as a secondary standard in analysis reducing the amount of highly toxic TCDD that was

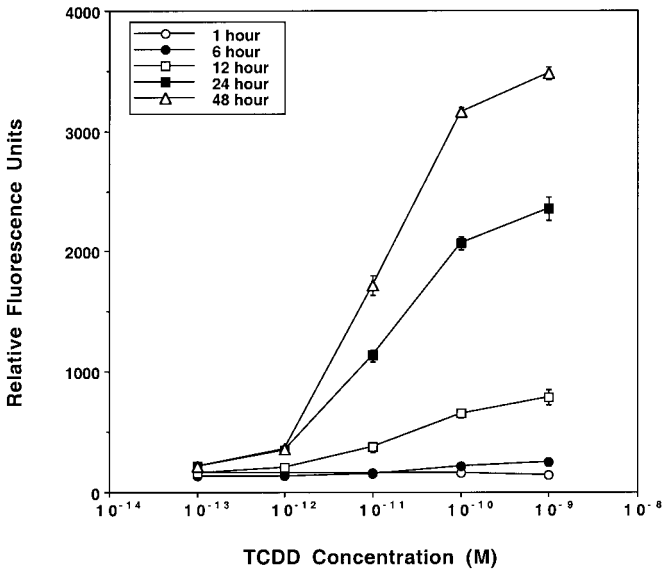


FIG. 6. Effect of incubation time on induction of EGFP gene expression by TCDD. H1G1.1c3 cells were treated with the indicated concentrations of TCDD at 33°C and the fluorescence was measured at the indicated time points, as described in Materials and Methods. Values represent the mean ± SD of 3 determinations. Induction of fluorescence was not significantly greater than control at any TCDD concentration at the 1-h time point; it became significantly greater than control at 10^{-10} M TCDD by the 6-h time point. For the 12-, 24-, and 48-h time points, it was significantly greater ($p < 0.05$, Student′s *t*-test) at concentrations at or above10^{-12} M TCDD.

TABLE 2
Comparison of the Concentrations for Half-Maximal Induction (EC_{50}) and Minimal Detection Limits (MDL) for TCDD in Various Recombinant Reporter-Gene Cell Bioassays

		EC_{50}		MDL	
Reference	Cell line	M	pg/well	M	pg/well
This paper	H1G1.1c3	1.84×10^{-11}	0.58	1.00×10^{-12}	0.03
Garrison *et al.*, 1996	H1L1.1c2[a]	2.00×10^{-11}	2.90	1.00×10^{-12}	0.15
Richter *et al.*, 1997	RTH-149	6.43×10^{-11}	2.59	1.00×10^{-12}	0.04
Murk *et al.*, 1996	H4IIE	1.00×10^{-11}	1.60	1.00×10^{-12}	0.16
Sanderson *et al.*, 1996	H4IIE	5.60×10^{-12}	0.45	2.40×10^{-12}	0.19
Postlind *et al.*, 1993	101L[a]	3.50×10^{-10}	NR	1.00×10^{-12}	NR

Note. NR. Not reported or calculable based on the information presented.
[a]Not microplate format.

used in assays. For this use, the surrogate standards needed to have some similarity to TCDD, but to be more rapidly metabolized and less biologically active. For some of the surrogates such as the 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (compound 4) the material had similar physical properties and chromatographic mobility to TCDD, but would be expected to be more rapidly metabolized. Accordingly, the relative potency of a series of TCDD surrogates was assessed by examining their ability to induce EGFP expression in the H1G1.1c3 cells. The structures of the 17 surrogates tested are shown in Figure 8. Dose-response experiments for the induction of EGFP by each of the surrogates were carried out and typical results for selected surrogates are shown in Figure 9. The EC_{50} for induction by all surrogates was determined from these curves and the induction equivalence factor (IEF) of each was calculated relative to that of TCDD (Figure 8). These results demonstrate the utility of this bioassay to provide a measure of the relative AhR-inducing potency of a given chemical. The results of our analysis reveal that surrogates with chlorines in the lateral R1 and R2 positions have the highest potency, while substitution of one chlorine at these positions, with other functional groups, reduced the potency by 96–11,000-fold, and substitution of both chlorines reduced the potency by 5,000–111,000-fold. In the case of surrogate number 1, the 76-fold decrease in activity can be solely attributed to the substituent present at point R3, since chlorines were present at positions 2, 3, 7, and 8 of the dibenzo-*p*-dioxin molecule. However, the addition of a bulky side group at position R3 does not appear to have as dramatic an effect on the biologic activity as when this same functional group is substituted at the R2 positions (compare surrogates 1 and 10). Previous studies have documented the critical importance of the lateral halogens in the biological AhR-dependent potency of TCDD (Safe, 1990). The other 2 most potent surrogates were numbers 4 (96-fold less potent than TCDD) and 6 (116-fold less potent than TCDD), which contained a methyl and an acetyl group, respectively, at position R2. Although methyl groups can often substitute for chlorine because of their similar size and hydrophobicity, the presence of these

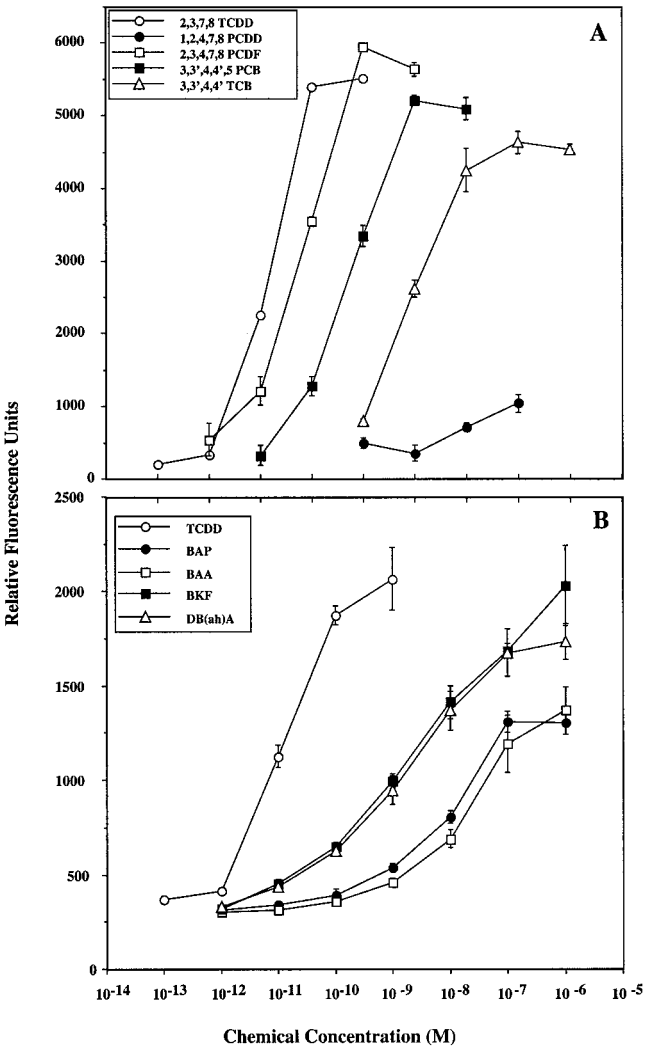
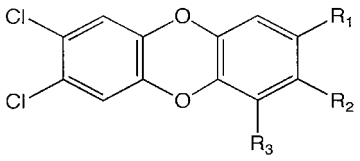


FIG. 7. Dose-response relationship for EGFP induction by HAHs and PAHs. H1G1.1c3 cells were incubated with the indicated concentrations of HAHs (A) and PAHs (B) for 24 h at 33°C, and the fluorescence was measured as described in Materials and Methods. Values represent the mean ± SD of 3 determinations.



Compound	R1	R2	R3	EC_{50}(nM)	IEF
TCDD	Cl	Cl	H	0.00894	1
Surrogate 1	Cl	Cl	$CHCHCOOC_2H_5$	0.684	0.0131
2	Cl	H	NH_2	4.48	0.00200
3	Cl	OH	H	31.3	0.000286
4	Cl	CH_3	H	0.860	0.0104
5	Cl	NH_2	H	1.77	0.00505
6	Cl	$COCH_3$	H	1.04	0.00860
7	Cl	$COOCH_3$	H	1.73	0.00517
8	Cl	$NHCOCH_3$	H	2.00	0.00447
9	Cl	COC_3H_7	H	2.99	0.00299
10	Cl	$CHCHCOOC_2H_5$	H	100	0.0000894
11	Cl	CHCHPhCOOH	H	14.8	0.000604
12	H	CH_3	H	107	0.0000836
13	H	OCH_3	H	145	0.0000617
14	H	$COOC_3H_7$	H	531	0.0000168
15	H	$CHCHCOOCH_3$	H	>1000	<0.0000089
16	CH_3	CH_3	H	45.7	0.000196
17	CH_3	$CHCHPhNH_2$	H	189	0.0000473

FIG. 8. Structures, EC_{50} and induction equivalency factors (IEFs) for 17 dioxin surrogates. IEFs were calculated by dividing the ED_{50} of TCDD by the ED_{50} of the surrogate.

in the R2 position reduces the inducing potency of the surrogates by about 100-fold.

The reduced inducing potency of these surrogates likely results from differences in the affinity of the individual surrogate for the AhR, and/or metabolism of the surrogate to a

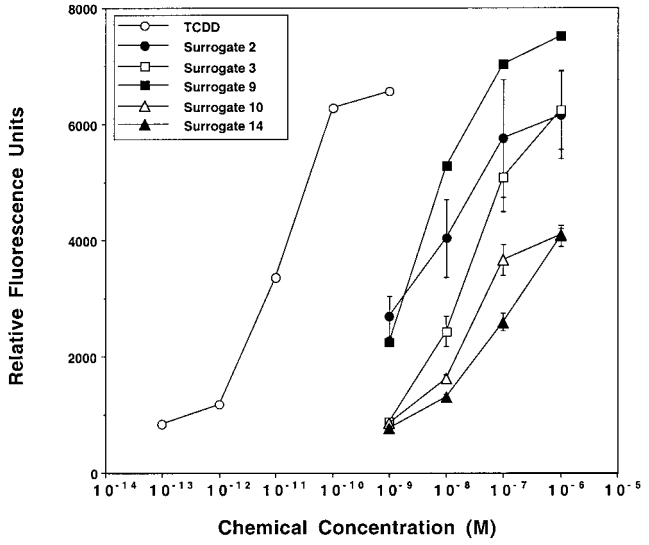


FIG. 9. Induction of EGFP by dioxin surrogates. H1G1.1c3 cells were incubated with increasing concentrations of the individual dioxin surrogates for 24 h at 33°C and the fluorescence measured as described in Materials and Methods. Values represent the mean ± SD of 3 determinations.

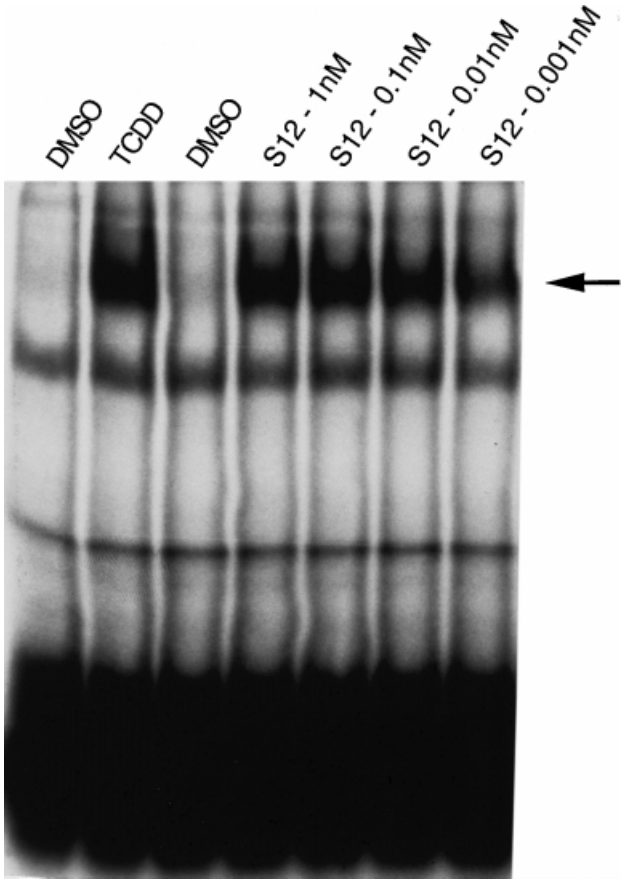


FIG. 10. Dose-dependent stimulation of AhR transformation and DNA binding by surrogate 12, *in vitro*. Guinea pig hepatic cytosol (16 mg/ml) was incubated with DMSO, 20-nM TCDD (in DMSO), or the indicated concentration of surrogate 12 (in DMSO) for 2 h at 20°C. Aliquots of each sample were mixed with [^{32}P]DRE and protein–DNA complexes resolved by gel retardation analysis, as described in Materials and Methods. The arrow indicates that position in the inducible AhR:DRE complex.

product with reduced AhR-binding affinity. To examine the ability of selected surrogates (5, 7, 12 and 13) to directly stimulate AhR transformation and DNA binding *in vitro*, and to assess their relative potency, we utilized gel retardation analysis. Figure 10 shows a concentration-dependent induction of AhR:DRE complex formation by surrogate 12. Phosphorimager analysis of these dose-response experiments (Fig. 11) revealed that these surrogates were relatively potent activators of AhR transformation and DNA binding, with EC_{50} values between 6–20 fold lower than that induced by TCDD (Table 3). Comparison of these *in vitro* potency results with the relative potency of each of these surrogates to induce luciferase reporter-gene expression in murine H1L1.1c2 cells after 4 hours of exposure or in H1G1.1c3 cells after 24 hours of exposure is shown in Table 3. These results reveal that the potency of each surrogate, relative to that of TCDD, was dependent upon the specific bioassay system used, with the chemicals being more potent in the gel retardation assay and least potent in the H1G1.1c3 cell bioassay. These results are

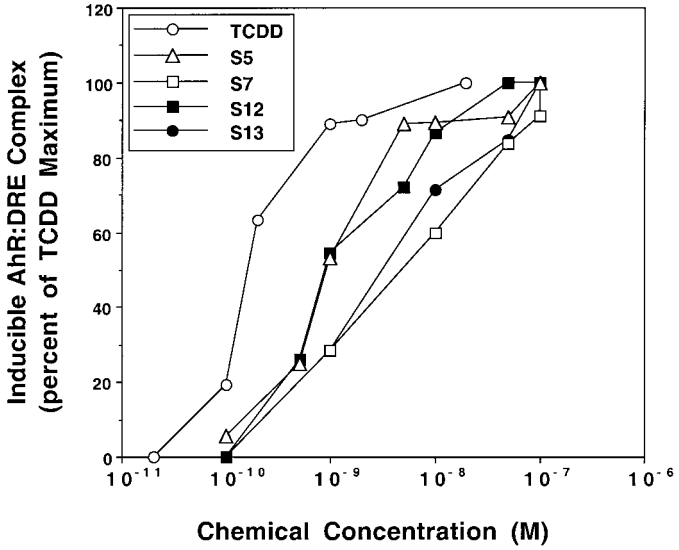


FIG. 11. Dose-dependent stimulation of AhR transformation and DNA binding by selected surrogates *in vitro*. Guinea pig hepatic cytosol (16 mg protein/ml) was incubated with increasing concentrations of TCDD or the indicated surrogate, protein–DNA complexes resolved by gel-retardation analysis and the amount of specific induced protein–DNA complex formation determined by phosphorimaging analysis, as described in Materials and Methods. The values are expressed as the mean ± SD of phosphorimager units of specific protein–DNA complex formed, determined from at least duplicate experiments.

consistent with the decreased potency in intact cells resulting from metabolism of the surrogate to a less bioactive form and the greater potency in H1L1.1c2 cells likely results from the difference in chemical incubation times (4 h for H1L1.1c2 cells and 24 h in H1G1.1c3 cells). Although the AhR-binding affinity and metabolic stability of these surrogates remains to be confirmed, our results do demonstrate the utility of our bioassay for in-depth QSAR analysis and AhR ligand design.

DISCUSSION

Numerous bioassays have been developed by our laboratory and others that take advantage of the AhR-dependent nature of TCDD (see Table 2). The majority of these bioassays contain a firefly luciferase-reporter gene under the control of DREs. These assays have been used to detect and quantify known AhR agonists in environmental samples, to estimate the biologic potency of complex mixtures of AhR agonists extracted from biologic matrixes, and to identify novel AhR agonists. The recombinant H1G1.1c3 cell line described herein has been stably transfected with a dioxin-responsive, EGFP reporter gene and is similarly able to detect such activity, but in a much more convenient and cost-effective manner, and without sacrificing sensitivity or selectivity for detection of AhR agonists. The data on TCDD chemical surrogates indicates that some of these materials could be used to standardize the EGFP reporter assay as secondary standards, to avoid the use of TCDD itself as a positive control. Some of these materials such as surrogate 4 could also be used as extraction standards for compounds of similar polarity to TCDD.

We previously examined the utility of the unmodified form of GFP as a reporter gene for assay development. In our hands, this form of GFP was a poor reporter in that little fluorescence was detected in transfected mammalian cells (data not shown). Given the difficulty in measuring GFP and its slow rate of formation in mammalian cells, we focused our attention on the EGFP reporter gene, which has been modified by mutations to optimize it for expression in mammalian cells (Clontech technical manual, PT2040). The EGFP gene also contains site-directed mutations in the chromophore that result in a red-shifted excitation spectra and a 4 to 35-fold enhancement in fluorescence brightness over that of wild-type GFP (Cormack *et al.*, 1996). Because EGFP is inherently fluorescent, it is not necessary to add enzyme substrates nor is it necessary to lyse the cells in order to measure reporter gene activity as is required for most common reporter genes such as luciferase, β-galactosidase, alkaline phosphatase, and chloramphenicol acetyltransferase. This aspect contributes to reductions in both reagent and labor costs. In addition, the ease of measuring EGFP in intact cells also has applications for time-course studies as reporter gene expression can be measured in "real time," as is also the case for the secreted alkaline phosphatase and human growth hormone reporter genes. The one potential disadvantage of EGFP is its reduced sensitivity due to lack of signal amplification (i.e., the ability of a given reporter to amplify the detected signal by continuous enzymatic cleavage of substrate). However, this has not proven to be a problem for this bioassay and the sensitivity and dynamic range of detection are comparable to luciferase.

The simplicity, rapidity, and cost effectiveness of measurement is the primary attribute of EGFP as a reporter gene. As

TABLE 3
Comparison of the Relative Potency (EC_{50}) of Selected Dioxin Surrogates

	GRA		H1L1.1c2		H1G1.1c3	
Surrogate	EC_{50}	RP	EC_{50}	RP	EC_{50}	RP
TCDD	0.15[a]	(1[b])	0.03	(1)	0.0089	(1)
5	0.9	(0.17)	0.3	(0.1)	1.77	(0.005)
7	4.4	(0.03)	2.0	(0.015)	1.73	(0.005)
12	0.9	(0.17)	150	(0.0002)	107	(0.00008)
13	3.0	(0.05)	75	(0.0004)	145	(0.00006)

Note. Relative potencies were determined from dose-response relationship studies using gel retardation analysis (GRA) with guinea pig hepatic cytosol and induction of luciferase for 4 h in H1L1.1c2 cells or induction of EGFP for 24 h in H1G1.1c3 cells.

[a] EC_{50} values were calculated from at least 2 dose-response curves.

[b] Relative potency (RP) values are expressed relative to that of TCDD (set to a potency value of 1).

such, the H1G1.1c3 cells provide a bioassay system appropriate for the rapid screening of large numbers of samples for dioxin-like activity. For example, the H1G1.1c3 cells could be used for rapid analysis of quantitative structure activity relationships (QSAR) of a series of chemicals in order to determine their relative potency and to define structural elements important for ligand binding. In fact, we have begun to utilize these cells for the rapid screening of combinatorial chemical libraries, in order to identify novel AhR ligands or classes of ligands for subsequent QSAR analysis (Nagy *et al.,* 2001). Environmental and product safety monitoring programs that also generate large numbers of samples will similarly benefit from this technology.

We demonstrate the suitability of these cells for chemical screening by determining the relative potency of a series of 17 previously uncharacterized dioxin surrogates to activate the AhR signal transduction pathway. To accomplish this, dose-response curves for each chemical were generated, which resulted in analysis of more than 200 individual measurements. Given the rapidity of the EGFP cell bioassay, it was possible for one person to complete these analyses in less than 3 h total time over a period of 3 days. Because assays are run concurrently, the number of samples that can be run in the same 3-day period can easily increase into the thousands. The lack of a requirement for expensive reagent addition also means that cost will not be a determining factor in deciding how many samples are to be screened (the cost for Promega luciferase reagent for a single 96-well assay is $0.2–0.3). In fact, the screening of the combinatorial chemical library, consisting of up to 12,090 chemicals, was completed by one person in our laboratory in less than one week. The total cost for this screening analysis using H1L1.1c2 cells would have been between $960 and $1400 for luciferase assay reagents alone. The reduction in person-hours needed for analysis by the EGFP, as compared to that of luciferase, is also a significant cost reduction. Thus, the H1G1.1c3 cells provide us with a relatively inexpensive and rapid high-throughput screening assay for AhR agonists, and it can provide useful mechanistic information regarding inducer stability when used in combination with gel retardation and luciferase cell bioassays.

There are numerous advantages to the EGFP cell bioassay as described above, but this screening system does have some minor limitations that can reduce assay utility. In contrast to our pGudLuc1.1-based H1L1.1c2 cell bioassay, which rapidly responds to AhR agonists (maximum induction at 4 h, Garrison *et al.,* 1996), longer induction times are required for EGFP induction in H1G1.1c3 cells. Consequently for those chemicals or biological responses that produce a rapid or transient activation of the AhR, the EGFP cell bioassay may be of limited utility, and in this instance, the pGudLuc1.1-based AhR-screening bioassay would be the system of choice. However there are only a few examples of such transient responses, and the majority of known AhR agonists are relatively stable inducers. Another potential limitation of the EGFP reporter system comes from test chemicals or chemicals present in complex mixtures, which could interfere with EGFP detection by their ability either to quench EGFP fluorescence or to fluoresce at the same excitation/emission wavelengths as EGFP. Several acridine-containing compounds that we examined interfered with the EGFP bioassay due to their high fluorescence. These were subsequently analyzed using a luciferase-based bioassay.

We observed that when the H1G1.1c3 cell line was maintained under selective pressure, inducible expression of EGFP remained high. However, when the cells were maintained in nonselective media we observed a gradual loss in TCDD-inducible EGFP expression. Accordingly, cells are maintained under constant selective pressure. This loss of responsiveness may result from the relatively high constitutive EGFP expression found in these cells. Previous researchers have reported that NIH3T3 cells expressing high levels of a stably transfected EGFP gene gradually lose EGFP expression over a period of months when not maintained under selective pressure, even when the cells were selected from a single clonal colony (Zeyda *et al.,* 1999). Although the reason for this loss is unknown, the relatively high levels of constitutive EGFP expression in H1G1.1c3 cells may put them at a competitive disadvantage, with high-expressing cells being outgrown by those with little or no constitutive EGFP. This reduction might also result from the instability of the plasmid vector at the genomic site of integration. Alternatively, it may be related to the reporter gene itself, since we have prepared cell lines stably transfected with a dioxin responsive luciferase construct (pGudLuc1.1) that have maintained their responsiveness for more than 10 years without any selective pressure. However, these cell lines express a much lower relative constitutive reporter gene expression than that of H1G1.1c3 cells. Analysis of additional EGFP stable cell clones may provide insight into this issue of instability.

Here we describe the development, optimization, and utilization of a recombinant cell line that responds to TCDD and other AhR agonists with the induction of EGFP expression in a time-, dose-, chemical- and AhR-dependent manner. The AhR-dependent nature of EGFP reporter gene induction and the rapidity of analysis have allowed for the detection and characterization of new AhR ligands. The H1G1.1c3 cell bioassay has the advantages of being rapid, low cost, and adaptable to high-throughput applications without sacrificing either sensitivity or selectivity. We therefore envision that this assay will be most suitable for adaptation to any application where cost or high sample numbers are an issue.

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