THIRD GENERATION CALUX LUCIFERASE REPORTER VECTORS - AMPLIFICATION OF DIOXIN RESPONSIVE ELEMENTS DRAMATICALLY INCREASES CALUX BIOASSAY SENSITIVITY AND RESPONSIVENESS

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Introduction

The development of accurate methods for the detection and quantitation of halogenated aromatic hydrocarbons (HAHs), such as polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) and related chemicals in environmental, biological, food and other matrices has been of critical importance given the ubiquitous distribution, resistance to biological and chemical degradation, high toxicity and potential for bioaccumulation/biomagnification of these chemicals. Accurate measurement of these chemicals in exposure matrices is necessary to allow proper epidemiological, risk assessment and exposure analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), the prototypical and most potent HAH, and related HAHs in human, animal and wildlife populations. While high-resolution instrumental analysis methods (GC/HRMS) are established for these chemicals and provide very accurate measurements of the HAH congeners of concern, these procedures are somewhat costly and time-consuming and impractical for rapid and large-scale screening analysis. Numerous bioanalytical methods (including bioassays and immunoassays) have been developed for the detection of these chemicals in extracts from a wide variety of matrices.[1,2] The majority of the available bioassay systems take advantage of the ability of the toxic HAHs of concern to bind to and activate the Ah (dioxin) receptor (AhR) and AhR signal transduction (i.e. gene expression). A major bioassay system that has gained widespread use and acceptance over the past 10 years is that of the CALUX (chemically-activated luciferase expression) cell bioassay.[1,2] These recombinant cells have been stably transfected with a luciferase reporter gene plasmid that contains four dioxin responsive elements (DREs, the DNA recognition site of the ligand (TCDD)-bound AhR) and which responsible for conferring AhR-responsiveness upon the luciferase reporter gene. These cells respond to TCDD, TCDD-like HAHs and related AhR agonists with the induction of luciferase reporter gene activity in a chemical-, dose-, time- and AhR-specific manner.[1,3] We previously reported on a second generation AhR-responsive CALUX luciferase reporter plasmid (pGudLuc6.1), that contained a more stable luciferase gene product that was targeted to cytosol, in contrast to our original AhR-CALUX reporter plasmid (pGudLuc1.1) which was targeted to peroxisomes and was more rapidly degraded.[4] Although cells stably transfected with the improved second generation CALUX reporter plasmid have been used extensively for HAH screening purposes, the limit of sensitivity and responsiveness of the reporter gene response in these cells at low TCDD/HAH levels is inadequate to allow them to be used for screening of small amounts of biological (i.e. blood) and/or other matrices (i.e. food) that contains relatively low levels of these compounds. Accordingly, the development of improved (i.e. more responsive and sensitive) CALUX cell bioassays is necessary to meet this application need. Here we describe the development and characterization of third generation AhR-responsive CALUX luciferase reporter plasmids that have dramatically improved sensitivity and reporter gene responsiveness to TCDD and related AhR agonists.

Materials and Methods

Generation of third generation AhR-responsive CALUX luciferase reporter plasmids. The development and testing of the AhR-responsive plasmid pGudLuc6.1 was described previously. Third generation DRE-responsive luciferase reporter plasmids containing increasing numbers of DREs were constructed by digestion of pGL7.0 immediately upstream of the MMTV viral promoter with BglII and ligation of the ligated/concatenated dioxin responsive domains (DRDs; 480bp BglII fragments from the upstream region of the murine CYP1A1 gene and each containing 4 DREs (excised from the plasmid pGudLuc6.1)) into the Bgl2 site of pGL7.0. Screening of the resulting clones by restriction digestion allowed identification of pGL7.0 plasmids containing between 1 and 5 DRDs inserted into the BglII site. The resulting plasmids were referred to as pGudLuc7.X with "X" representing the number of DRDs contained within the plasmid construct.

Transient and stable transfection, chemical treatment and luciferase analysis. Mouse hepatoma (Hepa1c1c7) cells were transiently transfected in 6 well plates with the indicated plasmid using transfectol (GeneChoice) and

allowed to incubate at 37°C for 24 hours. Cells were then with DMSO (0.1%) or the indicated concentration of TCDD in DMSO and luciferase activity determined as described in detail following another 24 hours of incubation.[4,5] Induction in stably transfected H1L6.1c2 (Hepa6.1) cells was included in each analysis as a positive control as this was the optimal second-generation AhR-responsive luciferase reporter plasmid. Mouse hepatoma (Hepa1c1c7) cells were stably transfected with the desired DRE-luciferase plasmid and pSV2Neo (a plasmid that constitutively expresses the neomycin resistance gene product) using lipofectamine and selection and isolation of stably transfected cell clones using geneticin (G418) was carried out as described.[2] Analyses of TCDD-dependent luciferase reporter gene induction in the stably transfected cell clones was carried out in cells grown in 96-well microplates as previously described using the the Promega luciferase assay system and an Anthos Lucy2 microplate luminometer.[5]

Results and Discussion

The CALUX cell bioassay, when combined with samples that have undergone extraction and clean-up, has been successfully used for the detection and relative quantitation of TCDD-like HAHs in numerous biological and environmental matrices and this method has been validated by several regulatory agencies for use as a screening bioassay for these chemicals.[1,2] While the current second generation CALUX bioassay based on the pGudLuc6.1 AhR-responsive luciferase reporter plasmid is adequate for screening of many matrices, this bioassay has some limitations when used to screen samples containing very low levels of AhR active HAHs or when the sample matrices is limiting (i.e. blood/serum samples). Accordingly, a more sensitive and/or more responsive CALUX bioassay would have application for use in screening of these low level/volume samples, in addition to the currently analyzed matrices. Amplification of the DREs within the CALUX reporter vector as well as amplification of the number of vectors in an individual cell and increasing the number of AhRs and/or the responsiveness of the AhR-responsive reporter gene promoter are avenues that can accomplish this. The current CALUX luciferase reporter plasmid contains 4 DREs (present in a single DRD) and increasing this by concatenation of the DRD and reinsertion of the concatamers into the vector has allowed us to generate a series of third generation CALUX luciferase reporter vectors containing between 4 and 20 DREs (i.e. between 1 to 5 DRD cassettes). We analyzed the TCDD-responsiveness of each of the plasmids that were prepared in mouse hepatoma (Hepa1c1c7) cells that were transiently transfected with individual vectors. While a wide range of responsiveness was observed in these transfection experiments, the overall magnitude of the induction response was dramatically increased as the number of DREs in the luciferase reporter plasmid was increased (Figure 1). Compared to pGudLuc6.1 transfected cells (the currently used second generation AhR-responsive reporter plasmid) where the magnitude of the induction response was ~7,000 relative light units per mg protein, the magnitude of the induction response with the concatenated DRDs ranged from ~10,000 to ~60,000 relative light units and TCDD dose dependency of the induction response was observed in each set of transfection results. Interestingly, the magnitude of the induction response with some plasmids with lower numbers of DRDs was comparable to those with higher numbers of DRDs. While this may be due to differences in sequence of each of the plasmids (due to spacing differences resulting from the varying numbers of DRDs (~500 bp each)) it is also very likely that there were significant differences in transfection efficiency of each plasmid in these experiments. The transfection efficiency was not determined in these studies since the relative functionality of the plasmids was the primary focus of these experiments. Interestingly, although the overall magnitude of the luciferase induction response (i.e. RLUs/mg protein) was dramatically increased with the plasmids containing more DRDs, determination of the overall fold induction produced by TCDD (comparison of control and TCDD induced activity) revealed that all of the plasmids (except that with 5 DRDs (figure 2)) had a similar fold induction response. This results primarily from the fact that as the overall magnitude of the induction response was increased, so too was the control luciferase activity, thus reducing the overall fold induction when normalized to the control (DMSO) results.

The above results not only demonstrate the functionality and increased response obtained of these new third generation, but they suggest that generation of new stably transfected cell lines containing these vectors would provide us with improved CALUX bioassay systems. Accordingly, mouse hepatoma (Hepa1c1c7) cells were stably transfected with selected vectors, cell clones isolated and their TCDD responsiveness determined. Two of the most responsive stable cell clones were grown up and further characterized. The TCDD concentration-response curves for two of the stable clones (tentatively designated as pGL7.2 (containing 2 DRDs - 8 DREs) and pGL7.5 (containing 5 DRDs - 20 DREs) are shown in Figure 3. The magnitude of the induction response for both new stable cell lines was dramatically greater than that of our currently used CALUX cell line (referred

to here as Hepa6.1), with the maximal luciferase activity for Hepa6.1 at ~14,000 RLUs, while that of pGL7.2 and pGL7.5 was 86,500 and 166,000 RLUs, respectively. In addition, one advantage of these new generation CALUX cell lines is the dramatically greater luciferase activity that is observed at lower inducer concentrations and this increased activity would not only allow more accurate determinations of the induction response at the lower end of the concentration response curve, but also results in a lower limit of detection with these new cells. Further characterization and optimization of these cell lines with regards to time course and temperature enhancing effects will further improve the new CALUX bioassay. In addition, the dramatically increased level of luciferase activity with the pGL7.5 cells suggests that it will be feasible to use these cells in a 384-well microplate format. Conversion to this format would not only reduce the amount of sample and reagents needed for cell growth and analysis, but also reduce analysis costs. Overall, these new cell lines will greatly increase the utility and number of applications of the CALUX cell bioassay system by increasing sample throughput and allow screening analysis of samples containing very low levels of AhR active HAHs and those where the sample matrices is limiting (i.e. blood/serum samples and samples from endangered species).

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Figure 1. TCDD-dependent induction of luciferase activity in Hep1c1c7 cells transiently transfected with the pGudLuc6.1 (containing 4 DREs), empty vector pGL7.0 (no DREs), or third generation vectors (containing between 4 (+1 DRD) and 20 (+5 DRDs) DREs). Values expressed as the mean RLUs ± range of duplicates.

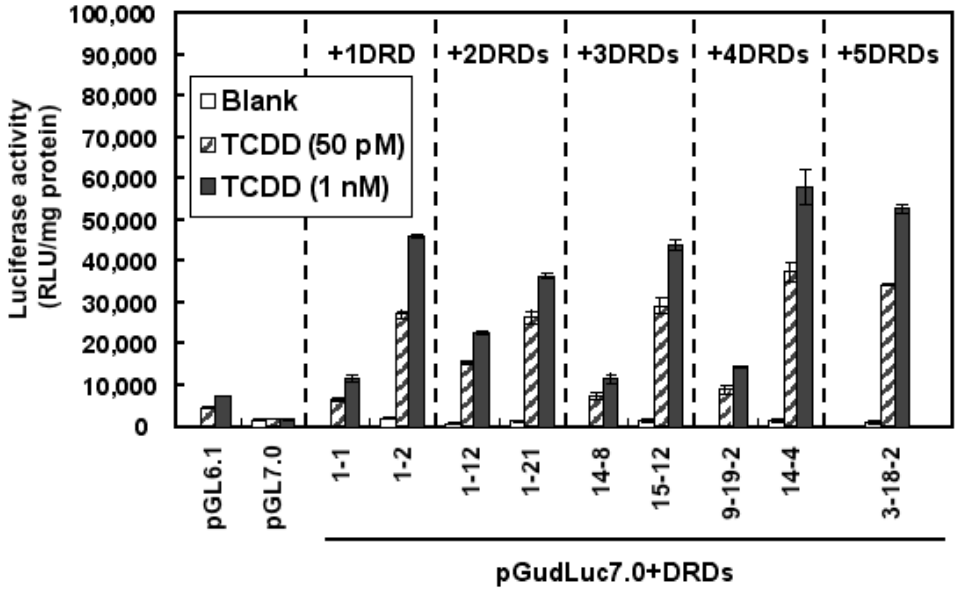

Figure 2. TCDD-dependent induction of luciferase activity in Hep1c1c7 cells transiently transfected with the second-generation CALUX plasmid pGudLuc6.1 (containing 4 DREs), empty vector pGL7.0 (no DREs), or one of five different third generation vectors (containing between 4 (indicated as +1 DRD) to 20 (indicated as +5 DRDs) DREs). Values are expressed as the mean fold induction relative to DMSO of duplicate samples.

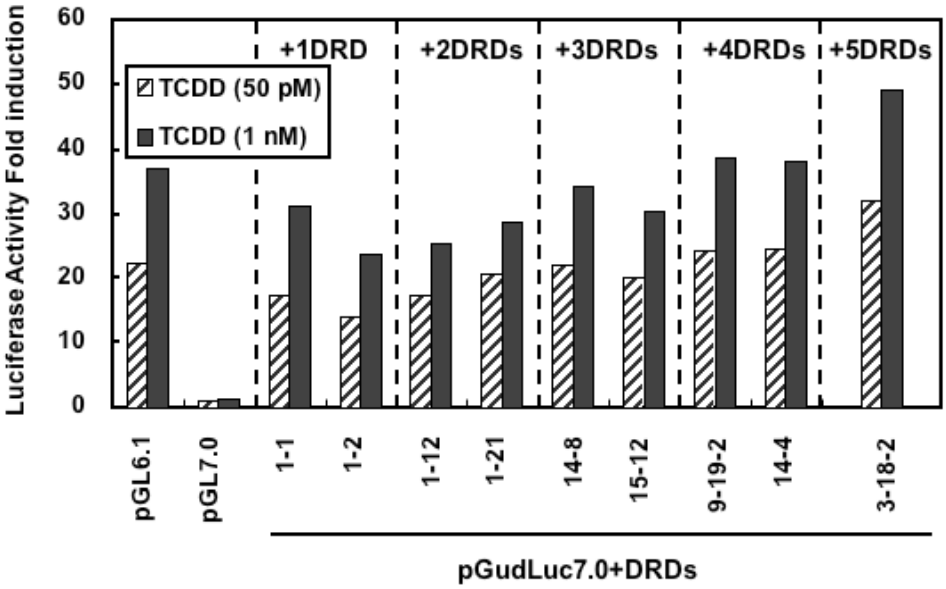

Figure 3. TCDD concentration-response curves for the induction of luciferase in Hep1c1c7 cells stably transfected with the second-generation CALUX plasmid pGudLuc6.1 (containing 4 DREs) or third generation vectors pGudLuc7.2 (containing 8 DREs) or pGudLuc7.5 (containing 20 DREs). Values expressed are the mean ± SD of triplicate samples after subtraction of control (DMSO) luminescence.