THE APPLICATION OF LOWERED INCUBATION TERMERATURE TO ENHANCE CALUX AND CAFLUX CELL BIOASSAY RESPONSES IN ENVIRONMENTAL ANALYSIS

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Introduction

Reporter genes produce a protein product in transfected cells that can be easily measured in intact or lysed cells and they have been extensively used in numerous basic and applied research applications.[1] Over the past 10 years, reporter gene assays have become widely accepted and used in epidemiological, risk assessment and exposure analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related dioxin-like halogenated aromatic hydrocarbons (HAHs) in the species of interest and in various exposure matrices (i.e. biological, environmental, food and feed) given that high-resolution instrumental analysis techniques are impractical for large scale screening analysis.[2] The majority of these bioanalytical systems take advantage of the ability of these chemicals to activate one or more aspects of the AhR-dependent mechanism of action. The most sensitive cell-based bioassay systems developed to date are CALUX (Chemically Activated Luciferase Expression) and CAFLUX (Chemically Activated Fluorescent Expression) bioassays, which utilize recombinant cell lines containing stably transfected dioxin (AhR)-responsive firefly luciferase or enhanced green fluorescent protein (EGFP) reporter genes, respectively.[2] Treatment of these cells with TCDD and related HAHs, other AhR agonists and extracts containing AhR agonists, results in induction of these reporter gene expression in a time-, dose-, AhR-, and chemical-specific manner and the level of reporter gene expression correlates with the total concentration of TCDD-like AhR inducers (agonists) in the sample.[2,3,4] While the current CALUX and CAFLUX bioassays are very sensitive, increasing their lower limit of sensitivity as well as magnitude of response and dynamic range for chemical detection would greatly increase their utility, particularly for those samples that contain low levels of dioxin-like HAHs (i.e. serum). The previous studies showed that temperature appears to be important on the activity of these two reporter genes, but very few analyzed the effect of cell growth temperatures on the overall activity of these reporter gene products in transfected mammalian cells. Here we have examined the effect of temperature on overall responsiveness of the reporter gene in CALUX and CAFLUX cells respond to HAHS and HAH containing extracts of environmental samples.

Materials and Methods

Chemicals**:** TCDD was generously provided by Dr. Steven Safe (Texas A&M University, College Station, TX). All solvents used for preparation of sediment sample were purchased from Fisher Scientific (Pittsburgh, PA). Cell culture reagents and media were purchased from Gibco/BRL (Grand Island, NY) and G418 was from Gemini Bio-Products (Woodland, CA).

Preparation of Environmental Sample and CALUX and CAFLUX Assays: Environmental sediment samples were extracted and cleaned up through a standard procedure to allow isolation of halogenated dioxins, furans and biphenyls.[5] Mouse hepatoma H1L6.1c2 and H1G1.1c3 were prepared and grown as previously described.[3,6] The stably transfected H1L6.1c2 cells, that contained the firefly luciferase gene under the control of 4 DREs, and the stably transfected H1G1.1c3 cells, that contained the EGFP (enhanced green fluorescent protein) gene under the control of 4 DREs.[3,7] Cells were incubated with chemicals or extracts of environmental sample for 24 hours at indicated temperature and assayed as previously described.[3,5,6] Condition for two incubators was set exactly the same except for the temperature, which was 33ºC and 37ºC.

Results and Discussion

In temperature range finding experiments, we previously found that 33ºC was the most optimal incubation temperature for TCDD to induce reporter gene expression in either H1L6.1c2 (luciferase) or H1G1.1c3 (GFP) cells. Here, we extended these studies to investigate whether reduced temperature (33ºC) would also improve CALUX and CAFLUX analysis when applied to environmental sample extracts. Sediment samples were collected and cleaned up in the lab followed the standard procedures to remove PAHs and other AhR agonists from the desired HAHs (dioxin like PCDD, PCDF and PCBs). The TCDD standard was run in parallel with environmental sample as a positive control and for comparative purpose. The results of TCDD dose response studies for luciferase (CALUX assay) at 37ºC and 33ºC are shown in Figure 1A and consistent with previous results, cells incubated at 33ºC exhibited significantly more luciferase activity than that at 37ºC. Not only was the luciferase activity at 33ºC, 5-15 fold greater than that observed in cells incubated at 37ºC at all TCDD concentrations, but it increased the amount of luciferase activity at the minimal detection limit of 0.45 pg TCDD/ml (compare 46.0 ± 19.2 relative light units (RLUs) at 33ºC to 1.3 ± 1.6 RLUs at 37ºC). Not surprisingly, the luciferase activity at 33ºC was similarly 5-15 fold greater than that observed in cells incubated at 37ºC when incubated with HAH containing sediment extracts (Figure 1B). Most significantly, the amount of dioxin or dioxin-like compounds extracted from less than 1 μg of sediment was essentially not detectable (produced less than 10 RLU over the solvent control) in the standard CALUX (37ºC) while they became easily detectable when incubated at 33ºC since they induced significantly higher luciferase activity (up to 300 RLU) over the solvent control. Interestingly, this small temperature change (from 37ºC to 33ºC) could significantly increase our bioassay's detection of TCDD-like compounds in environmental samples by up to 2 orders of magnitude. Similar results were obtained using the CAFLUX assay, where about a 2 fold greater amount of fluorescence activity from GFP was obtained from cells that had been incubated at 33ºC compared to those incubated at 37ºC with either TCDD standard or environment extract (Figure 1C & 1D). Not only was GFP fluorescence activity in cells treated with 0.45 pg TCDD/ml for 24 hours at 33ºC significantly higher than that observed from cells incubated at 37ºC, but the overall activity at 33ºC was significantly above background, while GFP activity at 37ºC was indistinguishable from background (Figure 1C). The GFP fluorescence activity induced by TCDD at 33ºC was ~2 fold greater than that observed in cells incubated at 37ºC at all concentrations of sediment extract (Figure 1D). We observed similar temperature-dependent differences in reporter gene activity (with optimum at 33ºC) using rat and human liver and guinea pig intestinal cell lines containing a stably

transfected AhR-responsive luciferase or EGFP reporter gene (data not shown), and in human ovarian and breast carcinoma cells containing an estrogen- or androgen-responsive luciferase reporter gene (data not shown). These results demonstrate that our observed temperature-dependence of reporter gene activity is due to an effect on the reporter gene itself, rather than an effect on single species or on the AhR signaling pathway itself. Here we have demonstrated that typical EGFP and firefly luciferase gene expression protocols in transfected mammalian cells for environmental analysis are not carried out at suboptimal temperatures necessary for production of maximal reporter gene activity. In those situations where optimal reporter gene activity in transfected mammalian cells is desired, cells should be incubated at lower temperatures (33ºC or optimal for the target cell line) during the gene expression period. This simple modification of gene induction protocols can lead to substantial increases in measurable reporter gene activity and therefore significantly increases the detectable limit and range of bioassay for dioxin and dioxin-like compounds in extract from environmental samples.

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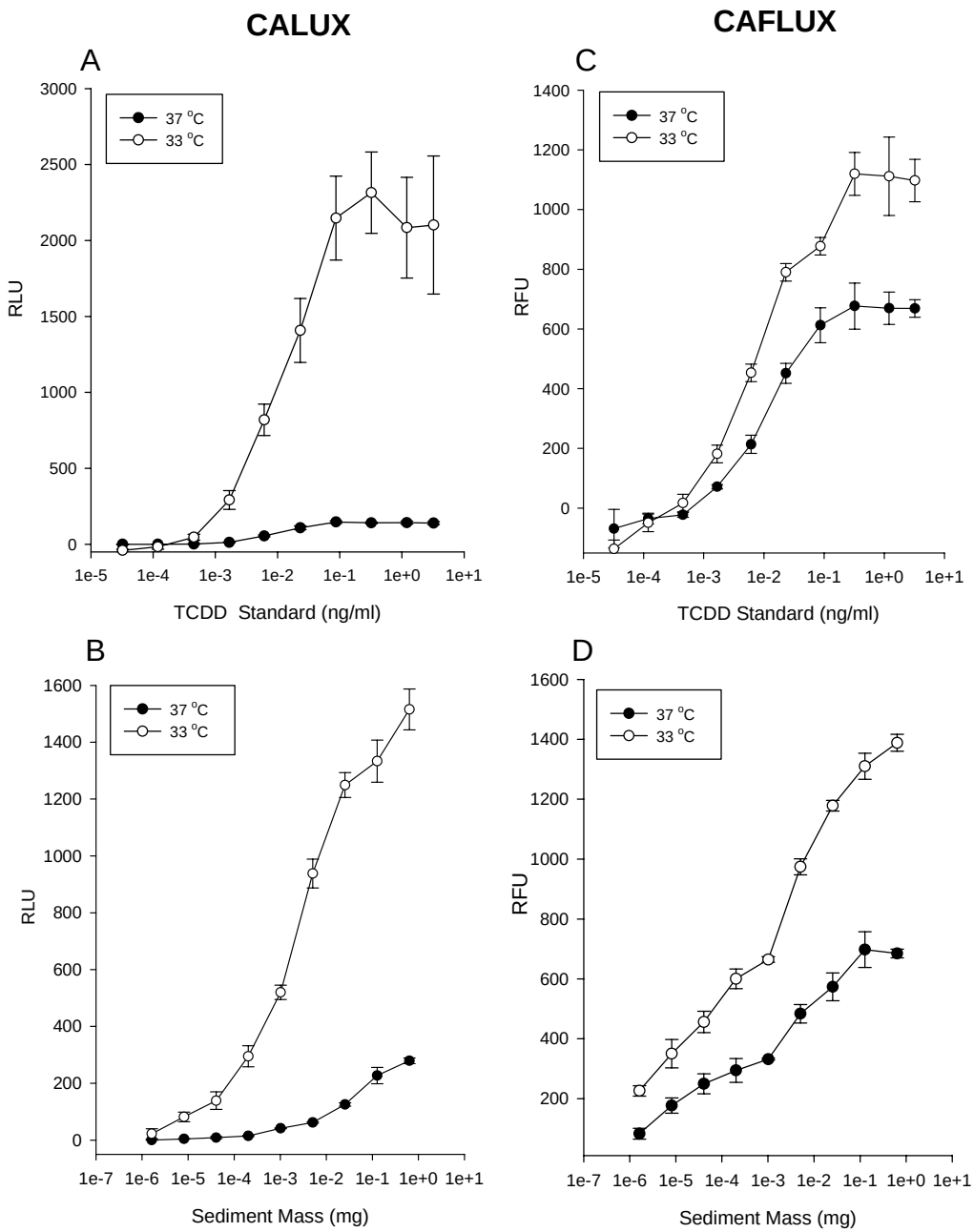

Figure 1. Temperature-dependent reporter gene expression in mouse CALUX (H1L6.1c2) and CAFLUX (H1G1.1c3) cell lines. H1L6.1c2 and H1G1.1c3 cells were incubated at the indicated temperature with either increasing concentrations of TCDD standard (A and C) or extract of environmental sediment sample (B and D) for 24 hours and luciferase and EGFP activity were determined as described. Values are expressed as relative light or fluorescence units (RLU and RFU) and represent the mean ± SD of triplicate determinations.