GENERATION OF DIOXIN RESPONSIVE CALUX H4IIE CELL LINES CONTAINING MULTIPLE DIOXIN RESPONSIVE ELEMENTS

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Introduction

Dioxins are persistent environmental contaminants that induce a wide variety of species- and tissue specific toxic effects. The mechanism of action of dioxin-like compounds involves their binding to the aryl hydrocarbon (Ah) receptor, followed by transportation of the toxin-Ah receptor complex into the nucleus of the cell and subsequent binding to specific sequences in the DNA, the dioxin responsive elements (DREs). The DR CALUX® bioassay is used routinely to detect dioxin-like chemicals in various biological matrices. The current DR CALUX® assay makes use of a reporter gene construct containing part of the mouse Cyp1a1 promoter (-1301 to -819), including four DREs, coupled to part of the MMTV promoter and the firefly luciferase gene[1]. Upon exposure to dioxin-like compounds, these cells express luciferase in a dose-dependent manner, which can be quantified using a luminometer. It has been suggested previously that the sensitivity and response can be improved by the integration of higher numbers of DREs into the plasmid[2,3]. Such an improved DR CALUX cell line could be useful for screening small amounts of biological matrices that contain low levels of dioxin-like compounds, such as blood. Here we describe the generation of several new reporter gene constructs, containing five tandem repeats of the mouse Cyp1a1 promoter, or 10 or 20 tandem repeats of a single DRE sequence. These multiple DRE constructs were tested in pGudLuc1 under control of the MMTV promoter, and in pGL2-tataluc under control of a minimal promoter. Here we mainly report on results using the 20 DRE-containing (pGudLuc1.5xCyp1a1) reporter construct.

Materials and methods

Construction of expression vectors. Several DNA fragments containing multiple DREs were synthesised by GeneArt (Regensburg, Germany): one fragment was composed of five copies of the -1313 to -820 part of the mCyp1a1 promoter (promoter ID 81793); since this part of the mCyp1a1 promoter contains four DREs, the synthesised quintuple repeat '5xCyp1a1' contains 20 DREs. Two other fragments '10xDRE' and '20xDRE' were composed of, respectively, 10 or 20 tandem repeats of a DRE sequence based on the -985 to -979 sequence of the rat Cyp1a1 promoter. All three fragments were cloned into pGudLuc1 (described previously by Garrison et al.[1]) and pGL2-tataluc (similar to pGL3-tataluc as described by Sonneveld et al.[4,5]). This resulted in six different plasmids (Table 1). These new plasmids were compared to similar constructs containing four DREs: the original DR CALUX plasmid pGudLuc1.1[1] and pGL2-tataluc.4xDRE (described previously as a pGL3-based construct[4,5]).

Transfections. The six new reporter gene constructs from Table 1 were first transiently transfected into mouse Hepa1c1c7 cells using calcium phosphate co-precipitation. Based on the results, pGudLuc1.5xCyp1a1 was selected for stable transfection. Rat hepatoma H4IIE cells were stably transfected with pGudLuc1.5xCyp1a1 and pSG5-neo using lipofectamine. A total of 176 G418 resistant clones were tested for their response to 0.1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin). Of the 21 TCDD-responsive clones, 6 were selected for further testing using a 0.03 - 300 pM TCDD curve.

CALUX bioassays. Cells were cultured in αMEM supplemented with 10% FCS. 50,000 cells/well were seeded in 96-wells plates in a volume of 100 µl. After 24h, 100 µl of αMEM containing the appropriate concentration of TCDD was added to the cells. 24h Later, the exposure medium was removed, cells were lysed and the luciferase activity was determined as described before[5].

Results and discussion

Transient transfection. All multiple DRE containing reporter gene constructs showed a dose-dependent response to TCDD. Fold inductions and response in relative light units (RLU) were similar for all constructs, and the EC50 values ranged from 5 to 13 pM (data not shown). Further studies were performed with stable clones.

Stable transfection. H4IIE cells were stably transfected with the new pGudLuc1.5xCyp1a1 construct, resulting in the isolation of 21 responsive clones (FI > 5) out of 176 tested clones. The six best clones were subjected to a concentration range of TCDD, and their responsiveness and sensitivity was compared to the original DR CALUX. The baseline RLU (at 0 pM TCDD) of four of the 5xCyp1a1 clones was 2-fold lower than of the original DR CALUX, while the maximum RLU (at 300 pM TCDD) of the 5xCyp1a1 clones ranged between 2-fold lower and 4-fold higher (Table 2). In general, the maximum- and baseline RLUs of the six new clones and the original DR CALUX were all in the same order of magnitude. However, the fold-induction was higher for all 5xCyp1a1 clones (1.5 to 20-fold). No changes in sensitivity were observed; all EC50 values except for clone #111 were in the range of 6 - 9 pM. Also the TCDD limit of detection (LOD, Table 2), was 0.3 pM for all cell lines, with the exception of clone #166 that had a slightly higher LOD. Of the six clones, clone #10 had the most favourable characteristics with respect to response (RLU), fold induction and sensitivity (Table 2, Figure 1).

In previous publications, He et al. constructed a similar 5xCyp1a1 reporter gene plasmid based on the third generation pGudLuc7, called pGudLuc7.5[2,3]. Their findings were that the fold induction did not increase in various cell lines, but the maximum and baseline RLU increased one to two orders of magnitude[3]. In Hepa1c1c7 cells the EC50 of the pGudLuc7.5 construct was similar to the second generation pGudLuc6.1 (4xDRE). However, they claimed that the minimal detection limit for TCDD was about two orders of magnitude lower for the 5xCyp1a1 construct. In contrast, we mainly observed an effect on the fold induction but not on the LOD. This may be caused by the fact that different cell lines were used for the experiments.

In conclusion, transfection of H4IIE cells with the newly constructed pGudLuc1.5xCyp1a1 resulted in a cell line with similar sensitivity, but five times higher fold induction compared to the current DR CALUX®. Although the detection limit was not improved, at TCDD concentrations > 1 pM the magnitude of response of the new DR CALUX line is significantly higher. This may facilitate accurate quantification in samples containing relatively low levels of dioxin-like compounds.

Table 1. Six new reporter gene constructs containing 10 or 20 DREs, and two 'control' constructs containing 4 DREs.

Construct	No. of DREs	Promoter
pGudLuc1.1 (DR CALUX®)[1]	4	MMTV
pGL2-tataluc.4xDRE[4,5]	4	minimal
pGudLuc1.5xCyp1a1	20	MMTV

pGL2-tataluc.5xCyp1a1	20	minimal
pGudLuc1.10xDRE	10	MMTV
pGL2-tataluc.10xDRE	10	minimal
pGudLuc1.20xDRE	20	MMTV
pGL2-tataluc.20xDRE	20	minimal

Table 2. TCDD inducible luciferase activity in H4IIE cells stably transfected with the original pGudLuc1.1 vector, and six stable clones containing the newly constructed pGudLuc1.5xCyp1a1.

Clone	Vector	Baseline (RLUx1000)[a]	Maximum (RLUx1000)[a]	Fold induction	EC50 (pM)	LOD (pM)
DR CALUX®	pGudLuc1.1	57.9 ± 4.0	1113.3 ± 15.3	19	8.9	0.3
5xCyp1a1#10	pGudLuc1.5xCyp1a1	20.7 ± 1.0	2076.7 ± 97.1	100	7.7	0.3
5xCyp1a1#15	pGudLuc1.5xCyp1a1	76.2 ± 3.2	3890.0 ± 169.7	51	7.6	0.3
5xCyp1a1#28	pGudLuc1.5xCyp1a1	18.0 ± 1.2	581.0 ± 69.7	32	6.3	0.3
5xCyp1a1#66	pGudLuc1.5xCyp1a1	18.9 ± 0.8	784.4 ± 21.1	41	8.4	0.3
5xCyp1a1#111	pGudLuc1.5xCyp1a1	1.5 ± 0.2	518.3 ± 43.4	345	21.1	0.3
5xCyp1a1#166	pGudLuc1.5xCyp1a1	31.8 ± 3.3	1556.7 ± 140.1	49	8.9	1.0

[a]Values are the mean ±SD of triplicate determinations

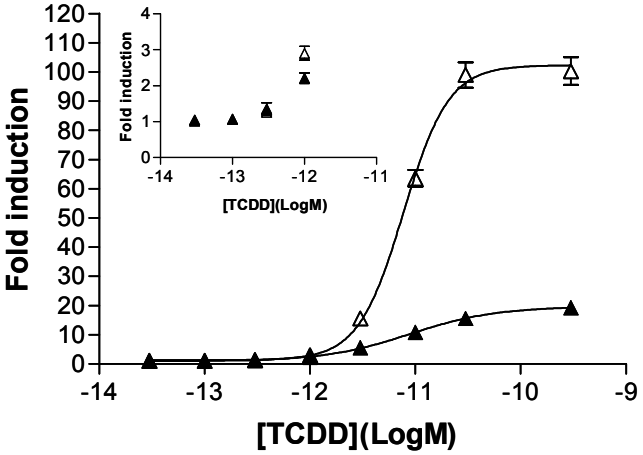

Figure 1. TCDD dose-response curves of the original DR CALUX® (closed triangles) and the new 20xDRE-containing stable cell line H4IIE-pGudLuc1.5xCyp1a1#10 (open triangles). Values represent the mean ±SD of triplicate determinations. The inset presents a zoom-in of the data points of 0.03 - 1 pM TCDD.

References:

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