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Dexamethasone inducible gene expression optimised by glucocorticoid antagonists

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Inducible expression of a specific target gene is a powerful tool to analyse factors involved in growth stimulation, cell cycle progression, oncogenic transformation or apoptosis. In an ideal system, the transfected gene should be *a priori* tightly repressed and strong induction should occur within a short time after stimulation. Conditional expression vectors based on endogenous mammalian control sequences from the metallothioneine gene promoter (1), heat shock factor promoter (2) and glucocorticoid responsive elements (3,4) have been reported (for review see 5). Drawbacks of these systems include leakiness at the inactive state and interference from other gene products by the induction conditions. Recently developed estrogen receptor–GAL4 fusion constructs exhibit high inducibility without side effects only in transient expression (6) but not in stably transfected cells (W. Mikulits, unpublished).

Here we describe an optimised protocol for dexamethasone inducible gene expression which lowers the basal activity of the hormone-dependent mouse mammary tumour virus long terminal repeat (MMTV-LTR) (7–9) by the antiglucocorticoid 17α-methyltestosterone (17α-MT) (10,11) prior to the induction with hormone. In addition, rapid de-induction of the conditionally elevated gene expression could be obtained in response to the novel antagonist ZK 112 993 (ZK).

To avoid inadvertent hormonal stimulation, all serum batches for cell culture were routinely treated with activated charcoal (12) to remove trace amounts of glucocorticoids. Cloning of full-length human thymidine kinase (TK) cDNA under the control of MMTV-LTR, stable transfection into mouse NIH 3T3tk$^-$ cells, selection under hypoxanthine–aminopterin–thymidine (HAT) and analysis of cytoplasmic RNA as well as TK enzyme activity was described previously (13). 17α-methyltestosterone was obtained from Fluka, ZK 112 993 [11β-(4-acetylphenyl)-17β-hydroxy-17α-(1-propinyl)-4,9-estradiene-3-one] from Schering AG and dexamethasone from Sigma.

The viral regulatory element in the MMTV-LTR can efficiently regulate transcription of any downstream target cDNA in response to the addition of glucocorticoids; most notably the synthetic hormone dexamethasone. This, in turn, allows to study effects of the corresponding gene expression in recipient cells after transfection (13,14). In our system, NIH 3T3tk$^-$ mouse fibroblasts were stably transfected with an expression vector carrying full length human TK cDNA as a reporter gene under the control of hormone inducible MMTV-LTR. This choice was favorable since TK mRNA and protein have a half-life of 2–3 h (15), whereas constructs containing chloramphenicol acetyltransferase as a reporter gene are compromised by high stability of the protein product. In a mixed pool of 50–100 transfectants we found that 30 μM of the glucocorticoid antagonist 17α-MT (Fig. 1A) lowered MMTV-LTR promoter basal activity 2.5-fold already 4 h after treating the cells with the antihormone (Fig. 1B). Flow cytometry of cells incubated for up to 96 h showed that this concentration of antiglucocorticoid did not affect growth or cell cycle distribution of transformants, whereas cells could be propagated indefinitely in the presence of 10 μM 17α-MT (data not shown). From the pool of transfectants, six independent single clones were chosen for detailed analysis. In all cases, the promoter leakiness was lowered by pre-incubating the isolated transformants for 6 h with 30 μM 17α-MT just prior to induction. Subsequently, cells were washed with phosphate buffered saline to remove the antagonist and induced with 1 μM dexamethasone. Levels of TK mRNA increased on average ~50-fold in the six individual clones (range 3–65-fold) and 25-fold in the pool of transformants (data not shown). The maximum of mRNA expression was obtained within 4 h of induction. Pre-treatment of cells with the antangonist did not alter kinetics of dexamethasone induction.

Similarly, the novel progesterone antagonist with antiglucocorticoid activity ZK 112 993 (ZK) already at 1–3 μM reduced basal promoter activity 2-fold (data not shown). Even at these low concentrations the substance effectively suppressed hormone stimulation by dexamethasone in co-cultivation experiments (Fig. 1C). Like 17α-MT, ZK had no influence on cell growth rates (data not shown). Taken together, these results indicate that both antagonists are valuable tools to suppress the leakiness of dexamethasone inducible gene expression. Subsequent hormone treatment results in rapid induction of a downstream target gene in fibroblasts.

Northern blot analysis of pooled clones demonstrated that S-phase specific genes were not affected by the exogenous hormone signal. Despite incubation with dexamethasone, transcription of endogenous S-phase specific genes for dihydrofolate reductase [DHFR] (16), β-actin (17), histone H4 (18) and the non-functional mouse TK of NIH 3T3tk$^-$ cells (19) was virtually absent in resting cells and activated only in the course of serum-dependent growth stimulation (Fig. 2). No interference of inducer or antagonists on endogenous promoters and gene products could be detected, which is an important functional characteristic for conditional gene expression.

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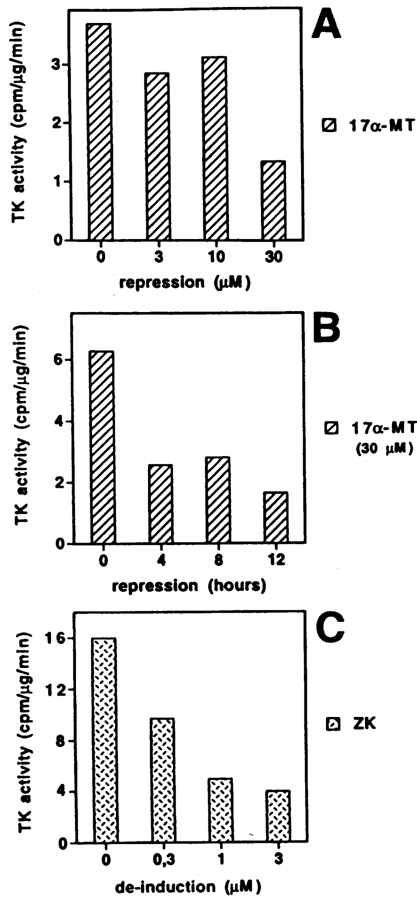

Figure 1. Reduction of basal activity by antagonist 17α-MT and de-induction of dexamethasone induced target enzyme activity by antagonist ZK. TK activity was measured in an exponentially growing pool of NIH 3T3tk⁻ mouse fibroblasts transfected with hTK cDNA under the control of the glucocorticoid dependent MMTV-LTR promoter. In untransfected cells, no TK activity was detectable. (**A**) Cells were incubated for 8 h with different concentrations of 17α-MT. (**B**) Treatment of cells with 30 µM 17α-MT for the times indicated. (**C**) Cells were stimulated for 7 h with 1 µM dexamethasone as agonist in combination with different concentrations of the antiglucocorticoid ZK.

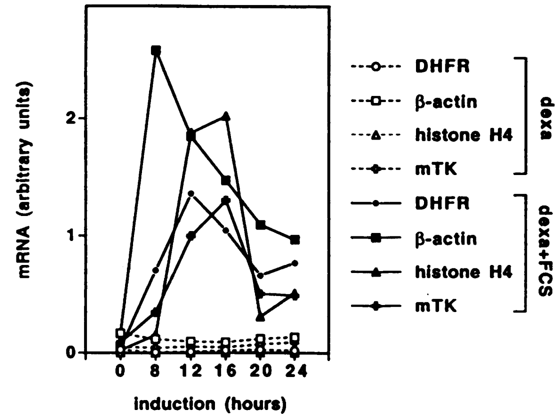

Figure 2. Expression of growth-dependent endogenous mRNAs in a pool of stably transfected NIH 3T3tk⁻ cells. Growth arrest in low serum (0.2% fetal calf serum; FCS) was followed by incubation of cells with 1 µM dexamethasone either alone or in combination with 10% FCS for the times indicated. Northern blots with cytoplasmic RNAs were hybridised sequentially with radiolabelled probes specific for DHFR, β-actin, histone H4 and the non-functional endogenous mouse TK mRNA. Subsequently, induction of these mRNAs was normalised to the constitutive β2-microglobulin transcripts (22) by laser densitometry. No difference of induction kinetics was observed for cells stimulated with serum alone. For reasons of clarity, the corresponding graphs were omitted.

A further benefit of the dexamethasone dependent system is the rapid induction kinetics: the highest target mRNA level is reached within 3–4 h. Therefore, this conditional expression is suitable, for example, for studying the decay of moderately stable products following de-induction *in vivo* or the effects of gene expression during distinct phases of the cell cycle. Unlike other systems in the literature (6,20,21), the antihormone/hormone-dependent transcription system described above requires only one round of transfection and selection. This may be a critical point for particularly sensitive or primary cells. Dexamethasone-dependent vectors also have a potential for future improvements by modifications in the hormone responsive elements of the LTR (9).

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