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ACTIVATION OF THE HSV-TK PROMOTER IN CONTROL REPORTER VECTOR pBLCAT5 BY LIGANDED NUCLEAR RETINOID RECEPTOR RXRa

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Abstract – Widely used reporter vector systems for studying the putative regulatory DNA elements usually contain basal promoters from pathogenic mammalian viruses. It is a common assumption that reliable results can be achieved only if the viral promoter activity is unaffected by *trans*-acting factors or any experimental treatment. Here we report that liganded nuclear retinoid receptor RXRα stimulates the HSV-TK promoter in control reporter vector pBLCAT5. Thus, TK driven reporter vectors should be employed only after thorough testing of the regulation of this promoter under experimental stimuli for a particular research purpose in order to avoid unreliable interpretation of the assay results.

Key words: HSV-TK promoter, pBLCAT5 vector, RXRα, *SOX3* gene

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INTRODUCTION

Control of gene expression is influenced at two levels: through modification of the chromatin structure of a gene locus and through the activity of *trans*-acting factors on local *cis*-regulatory sequences. In order to properly understand these complex mechanisms, a combination of so called "reductionist" approaches with *in vivo* analyses of the endogenous locus is required (McBride and Kleinjan, 2004).

"The reductionist" approach is based on genetic reporter systems that have been developed into an essential toolbox for studying *cis*-regulatory sequences as well as the roles of corresponding *trans*-acting factors. Regulatory sequences of interest are excised from their natural context and cloned into a vector, into which DNA sequences that code for an easily measurable enzyme are inserted and driven by the basal promoter. The enzyme serves as reporter of the function of cloned DNA elements controlling gene expression. Upon transfection of a reporter construct into mammalian cells and in response to various experimental stimuli (transcription factors, hormones, etc.), gene-controlling DNA elements modulate expression of the reporter gene (Ausubel *et al.,* 1997).

This powerful methodology for the study of gene expression and transcriptional regulation, aimed at revealing valuable information about the isolated control elements, has been in use for many years. Different reporter systems and assays were developed not only to make possible precise and sensitive measurements of transcription, but also to simplify the difficult and time-consuming process of identification of *cis*-regulatory sequences and transcription factors involved in gene expression regulation via these DNA elements.

The most commonly used reporter vector systems contain basal promoters from pathogenic mammalian viruses. They include the following promoters: the herpes simplex virus thymidine kinase (HSV-TK) (vectors: pBLCAT5, phRL-TK, phRG-TK); the simian virus 40 (SV40) (vectors: pCAT® 3, pGL3-Promoter, phRL-SV40, pSEAP2-Control, pβgal-Promoter); or cytomegalovirus (CMV) promoters (vectors: phRL-CMV, pCMVβ, pCMVGLuc). These viral promoters have been extensively used, since they have been reported to be constitutively expressed in most mammalian cell types.

The basic concept of the reporter system approach is that the reporter gene does not disturb metabolism of the transfected cells and that the gene is not endogenously expressed by the target cells creating background signals. Control reporter vectors are also often used as a standard to compare and to normalize for the *trans*-activating effi-

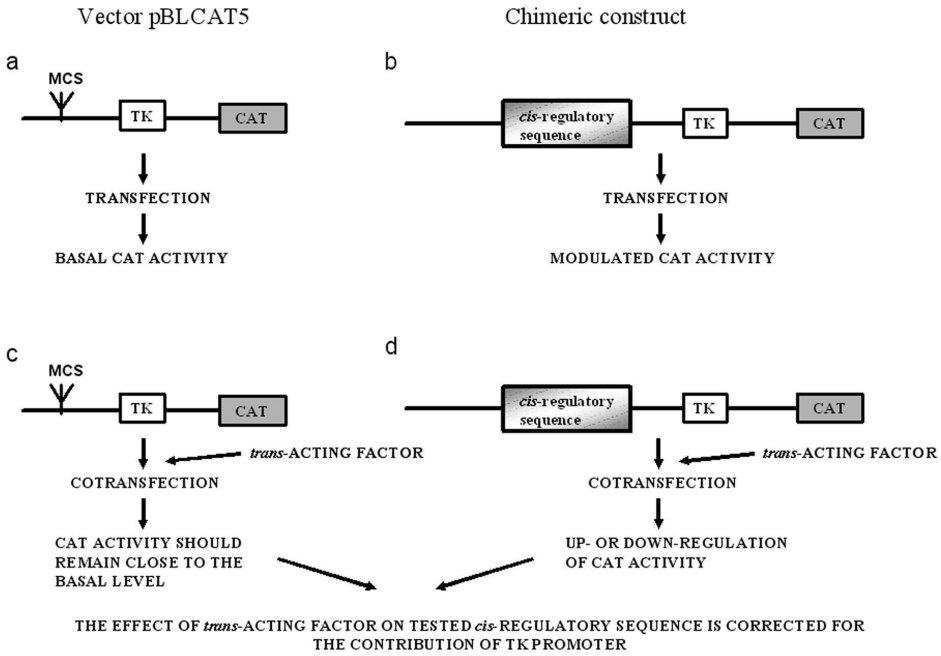

Fig. 1. Schematic overview of typical experimental approach for studying putative *cis*-regulatory sequences and *trans*-acting factors in regulation of gene expression. MSC - multiple cloning site, TK - herpes simplex virus thymidine kinase promoter, CAT - chloramphenicol acetyltransferase reporter gene.

ciency of different transcription factors. Therefore, one common assumption is that control vector expression is unaffected by *trans*-acting factors or any experimental treatment. Schematic illustration of a typical experimental approach is shown on Fig. 1, where the pBLCAT5 vector used in this study is presented as the control reporter system (B o s h a r t *et al.,* 1992). It contains the assayable chloramphenicol acetyltransferase (CAT) reporter gene from transposon Tn9 (A l t o n and V a p n e k, 1979) driven by the constitutively active viral promoter (HSV-TK) (M c K n i g h t *et al.,* 1981). The intrinsic activity of the insert-less pBLCAT5 vector represents basal CAT activity, providing an internal marker for monitoring reporter gene expression (Fig. 1a). The experimental chimeric construct containing *cis*-regulatory sequences of interest is assayed for the ability to modulate CAT reporter activity (Fig. 1b). In the next experimental step, cotransfection, where influence of *trans*-acting factor of relevance is studied, activity of the CAT reporter gene in the control vector should remain unchanged and close to the basal level (Fig. 1c). On the other hand, the effect of an ectopically expressed *trans*-acting factor in conjunction with the chimeric construct could be either up- or down- regulation of CAT activity related to the tested *cis*-regulatory sequence (Fig. 1d).

The main focus of our research was to study regulation of the expression *SOX* genes, a family of transcription factors involved in the control of diverse developmental processes. One particular aim was to study molecular mechanisms underlying retinoic acid (RA) induced transcriptional activation of the *SOX3* gene during neuronal differentiation of NT2/D1 embryonal carcinoma cells (S t e v a n o v i ć, 2003). Retinoic acid exerts its pleiotropic effects on cell growth and differentiation as ligand by the activation of two classes of nuclear retinoid receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (K a s t n e r *et al.,* 1995). Those receptors modulate transcription of target genes by interacting with specific *cis*-acting response elements (L a u d e t and G r o n e m e y e r 2002). Members of the nuclear retinoid receptor family have been used in many transfection studies to investigate their roles in the regulation of target gene expression.

Thus, in the course of our investigation focused on studying the regulation of *SOX3* gene expression by several nuclear receptor transcription factors, we employed the pBLCAT5 vector. Reporter vectors such as pBLCAT5 that contain the HSV-TK promoter are commonly used in transfection experiments aimed at studying the transcriptional activation mediated by nuclear receptors. Not only

do data from the literature indicate that the TK promoter is unresponsive to RA (B u s h *et al.,* 2003), but also no change in expression has been previously reported in co-transfection with the RXRα retinoid receptor expression vector in the presence or absence of RA (R o t t m a n *et al.,* 1991). However, the data presente in this paper demonstrated that in our control cotransfection experiments, liganded nuclear retinoid receptor RXRα significantly activated the CAT reporter gene driven by the TK promoter in the pBLCAT5 vector alone.

The results presented here emphasize that TK driven reporter vectors should be employed for *trans*-activating studies only after thorough testing of the regulation of this promoter under experimental stimuli for a particular research purpose in order to avoid misinterpretation or unreliable interpretation of the assay results.

MATERIALS AND METHODS

Cell culture

Maintenance of NT2/D1 cells was in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37 ºC in 10% CO_2. Neural differentiation of NT2/D1 cells was induced by addition of 10 µM all-trans retinoic acid (Sigma) for a period of 48 hours.

Transient transfection analysis

The day before transfection, a total of 1.2×10^6 NT2/D1 cells were seeded into a 10 cm dish. For each transfection 10 µg of pBLCAT5 or F20R12 construct, together with 2 µg of pBluescript (Stratagene) or pRS-hRXRα expression vector (M a n g e l s d o r f *et al.*, 1990) and 2 ìg of pCH110 vector (Amersham Pharmacia Biotech), were cotransfected using the calcium phosphate precipitation method (A u s u b e l *et al.*, 1997). A precipitate containing calcium phosphate and DNA was formed by slow mixing and gentle agitation of 0.25 M calcium chloride and solution containing DNA and 2 × Hepes-buffered saline solution (274 mM NaCl, 42 mM HEPES, 9.6 mM KCl, 1.5 mM Na_2HPO_4). Following 3-4 h of incubation, cells were washed twice with HEPES buffer (6.7 mM KCl, 142 mM NaCl, 10 mM HEPES) and fed fresh medium. After 48 h, cells were harvested and lysates were prepared by three cycles of freezing and thawing in 0.25 M Tris, pH 7.8, followed by centrifugaton. The pCH110 vector harboring the â-galactosidase gene was used as an internal control to normalize for transfection efficiency. Transfection quality DNA was isolated using the EndoFree® Plasmid Maxi kit (QIAGEN).

Repoter gene analysis

β-Gal assays were performed with a â-galactosidase enzyme assay system (Promega) and CAT activities were determined using CAT enzyme linked immunosorbent assay (CAT ELISA, Roche). The pBLCAT5 vector was used as a control in each transfection assay. Statistical significance was determined by the paired sample *t*-test using Analyse-it software for Microsoft Excel, and a difference of $p<0.05$ was considered significant.

Generation of CAT reporter construct F20R12

In order to generate the F20R12 construct, the fragment was amplified by PCR using primers containing *Hin*dIII and *Xba*I restriction enzyme sites in sense and antisense primer, respectively. Genomic clone X2.1H33 was used as template (S t e v a n o v i ć *et al.*, 1993). Primers for PCR amplification were as follows:

F20- 5' ctaagctTCGGTAATGATTGGCCAGGGCG 3' (-114)

R12-5' gatctagaATTCCCCGGGGTTGGGGCTTGGT 3' (-21)

Restriction sites are underlined in lowercase, while gene specific sequences are in uppercase. The numbers indicated in parenthesis correspond to the distance in nt from the 5' end of the sequence in uppercase to the *SOX3* transcription starting point (*tsp*).

Subsequent PCR amplification was done as follows: a denaturation step at 98 ºC for 1 min; 35 cycles of 98 ºC for 1 min, 51 ºC for 1 min, and 72 ºC for 1 min; and final extension at 72 ºC for 10 min. After digestion, the PCR product was cloned into the unique cloning sites (*Hin*dI-II and *Xba*I) of reporter vector pBLCAT5.

In silico analysis of HSV-TK promoter

The MatInspector Release 7.4.2 professional program (http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.pl) (Q u a n d t *et al.*, 1995) was used to search the matrix family library database to identify putative RXR binding sites in the HSV-TK promoter from the pBLCAT5 reporter vector. Only transcription factor binding sites that have a core similarity 1.00 are presented.

RESULTS AND DISCUSSION

We have previously reported that expression of the human *SOX3* gene is modulated during the RA-induced neuronal differentiation cascade of human teratocarcinoma cell line, NT2/D1 (S t e v a n o v i ć, 2003). Consequently, our aim was to localize control element(s) within the *SOX3* 5'- regulatory region that mediate a stimulatory effect of RA. For that purpose, series of fragments prepared by various 5' and 3' deletions of the *SOX3* 5' regulatory region were generated and cloned into the pBLCAT5 vector. The control, insert-less reporter vector, as well as the F20R12 construct in which the presumed *cis*-acting sequences from the *SOX3* gene were linked proximal to the basal HSV-TK promoter, were transfected and tested in NT2/D1 cells. Further, our cotransfection assays included RXR*α* as the representative of nuclear retinoid receptors, since it is well known that during development, RXR*α* is the primary and universal heterodimeric partner for other nuclear receptors that mediate RA activity (C h a m b o n, 1996). The CAT expression was assessed following transfection/cotransfection of NT2/D1 cells with described plasmids in the presence of retinoic acid.

We report here that the plasmid expressing RXR*α* transcription factor in the presence of retinoic acid increased CAT gene expression fromthe pBLCAT5 vector alone by a factor of 5.4 (RA + RXR versus RA, Fig. 2). Thus, unexpectedly strong activation of CAT expression from the control pBLCAT5 vector was observed in NT2/D1 cells in the presence of liganded RXR*α*.

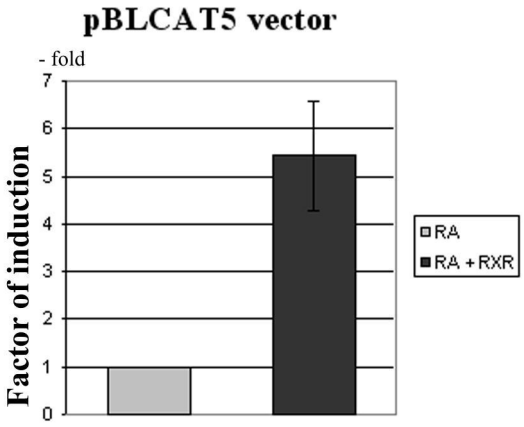

Fig. 2. Activation of CAT activity of pBLCAT5 vector by liganded RXRα. NT2/D1 cells were transfected with either insert-less pBLCAT5 vector alone, or together with RXRα expression plasmid in cotransfection experiments. The intrinsic CAT activity of pBLCAT5 in RA-induced NT2/D1 cells was arbitrarily set as 1 and the factor of induction due to the presence of RXRα was calculated and presented as the means ± S.D. of three independent experiments.

We further analyzed RA/RXR*α* transactivation of the F20R12 construct containing *SOX3* specific *cis*-regulatory elements cloned in pBLCAT5 (Fig. 3). Fragment F20R12 (-114 to -21 relative to the *SOX3 tsp*) caused 14.4-fold induction of CAT activity in the presence of liganded RXR*α* when data were not corrected for values of the control vector (uncorrected, RA + RXR versus RA, Fig. 3). The corrected value, when the CAT level of construct F20R12 was divided by the value of the pBLCAT5 control vector, revealed that the tested *SOX3* fragment displayed in fact only three-fold induction in cotransfection assay with liganded RXR*α* (corrected, RA + RXR versus RA, Fig. 3).

Although the level of CAT expression from the F20R12 chimeric construct was higher than in the control, exceptionally strong induction of the control pBLCAT5 plasmid substantially increased the background level, making it unreliable as a control. Thus, we are unable to consistently interpret our data, since the full effect of liganded RXR*α* on F20R12 could be masked under these circumstances.

Given the response of pBLCAT5 to RA/RXR*α* *trans*-activation as reported above, we speculated that retinoic acid response elements might be present in the

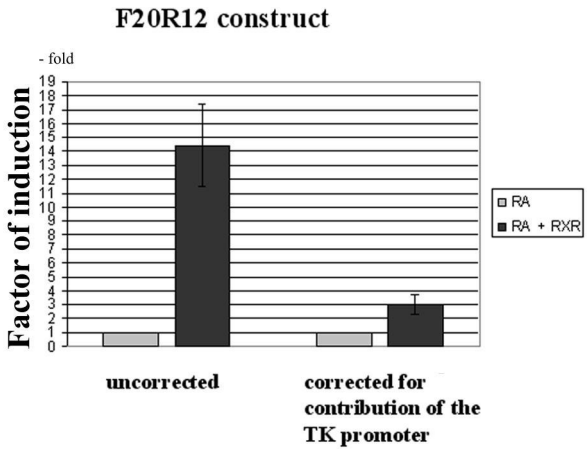

Fig. 3. Activation of CAT activity of F20R12 construct by liganded RXRα: NT2/D1 cells were transfected with either F20R12 construct alone, or together with RXRα expression plasmid in cotransfection experiments. The intrinsic CAT activity of F20R12 in RA-induced NT2/D1 cells was arbitrarily set as 1 and the factor of induction due to the presence of RXRα was calculated and presented as the means ± S.D. of three independent experiments. Data are presented as uncorrected and corrected for values of control vector pBLCAT5.

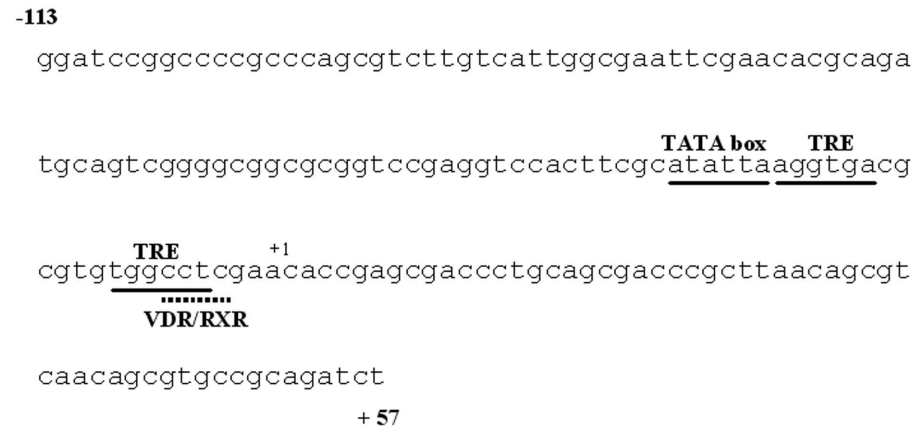

Fig. 4. MatInspector analysis of HSV-TK promoter nucleotide sequence. The putative VDR/RXR (vitamin D receptor/retinoid X receptor) binding site is underlined by a dashed line. The TRE (thyroid hormone response element) and TATA box are underlined by solid lines. The transcription starting point of the TK promoter is represented by +1. The TK promoter sequence was obtained from NCBI Database, GenBank accession no. 80483.

TK promoter driving the CAT reporter gene. We therefore performed MatInspector analysis to search for sequences conferring sensitivity to RXRα (potential RXR-binding sites) on the HSV-TK promoter sequence from the pBLCAT5 vector. Examination of this promoter region revealed the presence of a putative VDR/RXR heterodimer-binding site downstream of the TATA box (Fig. 4). This potential element overlaps the second half-site previously found to bind thyroid hormone receptor (P a r k *et al.*, 1993) and may be responsible for transcriptional induction of the HSV-TK promoter by RXR*α* in the presence of retinoic acid. We therefore hypothesize that this putative VDR/RXR binding site could contribute to the observed increase in background CAT activity.

Viral promoters commonly used in reporter vector systems have been studied for the presence of transcription factor binding sites enabling them to aquire information regarding potential "cross-talk" with other enhancers/promoters. The CMV promoter has been reported to contain many transcription factor binding sites (e.g., two AP2; four NF-kappaB; and five CREB binding sites) (M e i e r and S t i n s k i, 1996), and its activity is therefore likely to be affected by some experimental stimuli. However, such data regarding the HSV-TK promoter are scarce and have started to accumulate only recently.

For example, activation of the HSV-TK promoter by orphan nuclear receptors has been reported (M a t u s z y k *et al.*, 2002). In the analysis of Fas Ligand promoter-driven transcription, the pRL-TK plasmid (HSV-TK promoter upstream of the native *Renilla* luciferase gene) was used as an internal control of transfection efficiency. This study showed that luciferase expression was enhanced by cotransfection with vectors expressing orphan nuclear receptors belonging to the Nur77 family (Nur77, Nurr1, Nor-1), thus leading to false interpretation of results concerning the effect of Nur77 family proteins on the experimental reporter system (M a t u s z y k *et al.*, 2002).

Members of the nuclear receptor family are not the only transcription factors that can enhance expression driven by the HSV-TK promoter. It has been reported that the human GATA-6 transcription factor increased *Renilla* luciferase gene expression from vector pRL-TK, used as transfection normalization control (H o and S t r a u s s, 2004). This alteration of control reporter gene activity by the tested *trans*-acting factor was shown to cause erroneous normalization of transfection efficiency and thus misinterpretation of results in a *trans*-activation assay.

Also, research conducted by B e t r a b e t *et al.,* (2004) casts doubt on the reliability of using viral promoters (HSV-TK in particular) in a cell-based bioassay for measuring bioactivities of drugs and hormones for human therapy. To be specific, the reporter vector containing PRE (progesterone response element), pPRE-tK-CAT, transfected into a human breast cell line and induced by sequential doses of progesterone, did not show the dose-dependent consistency in expression profile that is the vital determinant of reliability in the function of a reporter gene promoter in receptor/reporter assays.

The results presented here show that nuclear retinoid receptor RXRα, when activated by its ligand, retinoic acid, stimulates transcription driven by the HSV-TK pro-

moter in control reporter vector pBLCAT5. Our data indicate that studies with the TK promoter used to analyze and compare the activities of RXRα response elements need to be interpreted with caution, since the putative VDR/RXR-binding site within the TK promoter might interfere with activity of the tested element. In addition to our data, other reported results with the use of various cell lines, expression vectors, and treatments strongly indicate that there is a disadvantage to using TK-driven reporter vectors, since expression may be influenced by various experimental conditions (http://www.promega.com/enotes/faqspeak/9912/fq0013.htm).

In conclusion, here report that liganded transcription factor RXRα stimulates gene expression of the CAT reporter gene driven by the TK promoter in control vector pBLCAT5. These data highlight the need to monitor variations in control gene expression arising due to experimental treatment in cotransfection studies. Taken together, these findings emphasize the importance of selecting an appropriate control reporter plasmid for the normalization of *trans*-activating efficiency.

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ЛИГАНДОМ ИНДУКОВАНИ НУКЛЕАРНИ РЕЦЕПТОР RXRa АКТИВИРА HSV-TK ПРОМОТОР У КОНТРОЛНОМ РЕПОРТЕРСКОМ ВЕКТОРУ pBLCAT5

ГОРДАНА НИКЧЕВИЋ, НАТАША КОВАЧЕВИЋ ГРУЈИЧИЋ и МИЛЕНА СТЕВАНОВИЋ

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Векторски системи који садрже репортерске гене су широко коришћени за испитивање регулаторних ДНК елемената. Промотори патогених вируса сисара најчешће се користе као базални промотори у овим репортерским системима. Опште је прихваћена претпоставка да се поуздани резултати могу добити само под условом да на активност вирусног промотора не утичу фактори који делују *in trans* или било који други примењени експериментални третмани. У овом раду смо показали да лигандом индуковани нуклеарни рецептор RXRα активира HSV-TK промотор у контролном репортерском вектору pBLCAT5. На основу приказаних резултата може се закључити да векторске системе који имају TK као базални промотор треба користити само после детаљног тестирања регулације експресије овог промотора. При томе је неопходно применити експерименталне услове специфичне за поједина истраживања да би се избегле погрешне интерпретације резултата.