Controlled expression of enhanced green fluorescent protein and hepatitis B virus precore protein in mammalian cells*

ZHU Xiaodong[1], YAO Feng[2] and TIEN Po[1]**

(1. Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China; 2. Laboratory of Tissue Repair and Gene Transfer, Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA)

Received July 24, 2002; revised August 23, 2002

Abstract A novel tetracycline regulation expression system was used to regulate the expression of enhanced green fluorescent protein (EGFP) and hepatitis B virus precore protein in the mammalian cell lines with lipofectAMINE. Flow cytometry assays showed that application of the system resulted in about 18-fold induction of EGFP expression in CHO cell lines and 5-fold induction in SSMC-7721 cells and about 2-fold in the HEK293 cells. Furthermore, the effective use of this system for the controlled expression of HBV precore protein gene in hepatocellular carcinoma cells was tested.

Keywords: tetracycline inducible expression system, enhanced green fluorescent protein, hepatitis B virus, transdominant negative proteins.

The problems that investigators have to solve in gene therapy are obtaining an efficient delivery system, achieving a regulatable gene expression and the shortage of knowledge of the genes to be delivered. Tetracycline inducible expression system, named Tet-off and Tet-on, is the most applicable and the only system for reversibly controlled gene expression in mice[1~4]. But it also offers potential pleiotrophic effects, high basal levels of expression and toxicity to cells caused by the mammalian cell transcription factor in the fusion protein. In order to improve that, the tetracycline-regulated expression system (T-Rex) was constructed and proved to be a more efficient vector which made accurate expression of transfected genes possible. In this study we analyzed the regulatable expression of enhanced green fluorescent protein (EGFP) in different types of mammalian cells by using this system and flow cytometry, and also studied reversible expression of hepatitis B virus precore protein, a transdominant negative protein, in the hepatocarcinoma cell line.

1 Materials and methods

1.1 Plasmids, cells and primer design

The plasmids pCMVtetOEGF, pCDNA3-tetR expressing tetracycline repressor (tetR) and pCDNA3 (as the control) were kindly supplied by Harvard Medical School. Plasmid TOPO-HBV containing HBV genome was constructed by Professor Gao Guangxia at Institute of Microbiology, Chinese Academy of Sciences; pCMVEGFP was obtained from Clonetech (USA). To construct pCMVtetOprecore, a pair of primers, 5′- TTGGATCCATGCAACTTTTTCACCTCT-3′ with a *Bam*HI restriction site and 5′-TTTGCGGCCGCTAACATTGAGGTTCCCG-3′ with a *Not*I restriction site, was designed. Chinese hamster ovary cells (CHO k1) and human embryo kidney cells (HEK 293) were obtained from Cell Bank of Peking Union Medical College; human hepatocarcinoma SSMC-7721 cells were obtained from Cell Bank of Chinese Academy of Sciences.

1.2 Cell culture

SSMC-7721 cells were grown in RPMI 1640 medium supplemented with 2 mmol/L L- glutamine, and 10% fetal bovine serum (FBS). CHO K1 cells were grown in F12 medium containing 10% FBS. HEK 293 cells were grown in DMEM medium containing 10% FBS.

1.3 Transient transfection assays

All plasmids were purified using a plasmid Maxi kit (QIAGEN). All cells were seeded at a density of $1\times10^5 \sim 2\times10^5$ cells per well in 6-well cell culture plates. At 20 to 24 h of culturing, cells were trans-

* Supported by the Major State Basic Research Development Program of China (G1999075602)

** To whom correspondence should be addressed. E-mail: tienpo@sun.im.ac.cn

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

fected with either pCMVtetOEGFP or pCMVtetO-precore in the presence of pCDNA3 as control or pCDNA3-tetR by lipofectAMINE-mediated transfection with normal growth medium in the presence or absence of tetracycline (5 μg/mL). Cells were harvested at 48 h posttransfection. Tranfections were typically performed according to the protocols of Gibco BRL company (USA).

1.4 Isolation of 7721-tetR clones expressing tetR

SSMC-7721 cell line was transfected with 5 μg of *Xho* I- linearized pCDNA3-tetR. After 48 h of transfection, cells resistant to G418 were selected by a medium containing G418 at concentration of 400 μg/mL. The tetR-expressing cell lines were determined by enzyme-linked immunosorbant assay (ELISA).

1.5 FACS analysis of transfected cells

The transfected cells were collected from plates using trypsin-EDTA and washed three times with PBS, then analyzed by flow cytometer (Becton Diskon, USA).

1.6 Immunoblotting

For immunoblotting, cells were scraped into PBS and pelleted by low centrifugation after 48 h posttransfection. Pellets were then incubated in a lysis buffer overnight at 4 ℃. SDS-PAGE was performed following standard procedures on a 12% polyacrylamide gel. Proteins on the gel were eletroblotted to nitrocellulose membranes and reacted with antibody against HBV core antigen and then detected by chemiluminescence (ECL, Amersham).

2 Results

2.1 Construction and expression of tetracycline inducible plasmids in mammalian cells

To generate the plasmid pCMVtetOEGFP, the 611 bp *Bam* HI/*Not* I fragment of pCMVtetOEGF was replaced by a fragment of pCMVEGFP containing the EGFP open reading frame. The construct was then transfected by LipofectAMINE into SSMC 7721 cells. The expression of EGFP was viewed by a fluorescence microscope. Plate I demonstrates the typical green fluorescence in SSMC 7721 cells. In addition, we amplified the HBV precore protein gene (640 bp) by PCR and constructed plasmid pCMVtetOprecore by inserting the amplified DNA fragment into the tetracycline inducible plasmid pCMVtetO. This construct, pCMVtetOprecore was transfected into SSMC-7721 cells. Fig. 1 demonstrates the presence of a 22 kD protein band by Western blot analysis using the antibody against HBV core antigen, which is consistent with the previous report[5].

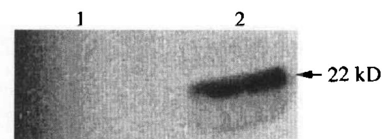

Fig. 1. Western blot analysis of HBV precore protein expression in SSMC-7721 cells. Protein from total cellular extracts of cells was probed with non-specific mouse antibody (lane 1), or antibody to HBV core antigen (lane 2).

2.2 EGFP expression under inducing and repressing conditions

To investigate the capacity of the tetracycline-regulated expression system in regulating gene expression in mammalian cells, we chose three types of cells: CHO k1 cells, SSMC-7721 cells and HEK 293 cells. Cells were independently transfected with (1) pCMVtetOEGFP (0.5 μg) and pCDNA3 (2 μg); (2) pCMVtetOEGFP (0.5 μg) and pCDNA3-tetR (2 μg); and (3) with pCMVtetOEGFP (0.5 μg) and pCDNA3-tetR (2 μg) in the presence of tetracycline (5 μg/mL). Cells were harvested 48 h posttransfection then subjected to FACS analysis. The mean fluorescence intensity(MFI) of the transfected cell populations was used as a measure of EGFP reporter gene expression. The results presented in Fig. 2 show that MFI of the EGFP-positive cell population and cells transfection efficiency depend on different conditions used. As shown in Fig. 2(b) and 2(c), the MFI of CHO K1 cells co-transfected with the pCMV-tetOEGF and pCDNA3 was 809.5, about 20-fold higher than that of CHO K1 cells which were co-transfected with the pCMVtetOEGFP and pCDNA3-tetR and the latter had MFI of 42.2. This indicates that the EGFP expression was repressed efficiently due to the existence of the tetracycline repressor. As shown in Fig. 2(d), when induced by tetracycline, EGFP mean fluorescence intensity of CHO K1 cells was 771.6, which is almost to the level of the cells containing only the reporter construct, indicating that the tetR-mediated repression can be efficiently released by the presence of tetracycline (5 μg/mL) with the same transfection efficiency. In addition, the cells transfected with either pCDNA3-tetR or pCDNA3 showed the same fluorescence intensity which was not

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

influenced by the presence of tetracycline (data not shown).

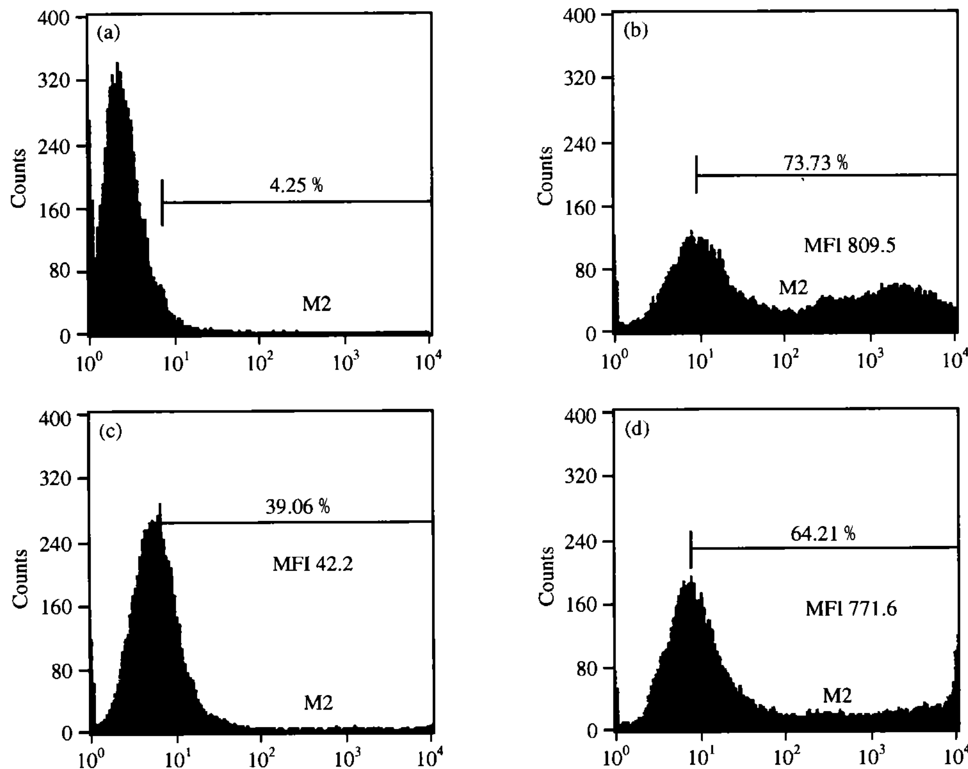

Fig. 2. Flow cytometric analysis of controlled EGFP expression in CHO K1 cells. (a) Untransfected; (b) pCMVtet OEGFP (0.5 μg) and pCDNA3 (2 μg) transfected; and (c) pCMVtetOEGFP (0.5 μg) and pCDNA3-tetR (2 μg) transfected; (d) pCMVtetOEGFP (0.5 μg) and pCDNA3-tetR (2 μg) transfected in the presence of tetracycline (5 μg/mL).

With the same procedure, we performed FACS for SSMC-7721 cells and HEK 293 cells to test if the findings we found in CHO K1 is a phenomenon in the tetracycline-dependent regulation of EGFP gene expression. The results presented in Table 1 show that MFI of the transfected cells varies depending on different cell lines. TetR can produce respectively 5.4-fold and 2-fold repression in SSMC-7721 cells and HEK293 cells. On the other hand, in the presence of tetracycline, the MFI values increased respectively more than 5-fold and 1.75-fold in 7721 cells and HEK 293 cells in that tetR was inhibited by tetracycline.

Table 1. FACS analysis of controlled EGFP expression in two cell lines

Plasmid	Tetracycline	MFI	
		SSMC-7721	HEK 293
pCMVtetOEGFP and pCDNA3	−	155.7	83.5
pCMVtetOEGFP and pCDNA3tetR	−	28.8	40.0
PCMVtetOEGFP and pCDNA3tetR	+	145.5	70.0

2.3 Transient transfection assays of pCMVtetOprecore and the regulated effect of pCMVtetOprecore in cell lines stably expressing tetR

The precore protein of human hepatitis B virus (HBV) functions as transdominant negative polypeptides that would inhibit HBV replication. We cloned precore protein gene of HBV into the pCMVtetO vector and transfected pCMVtetOprecore into SSMC-7721 cells. Western blot analysis of the cell extracts showed that HBV precore protein could be expressed and the expression was repressed by tetracycline repressor (TetR), and the repression can be efficiently released by the presence of tetracycline (Fig. 3).

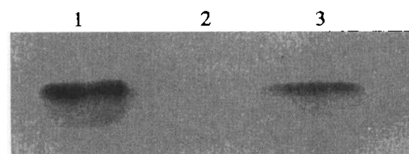

Fig. 3. Tetracycline regulation of pCMVtetoprecore in SSMC-7721 cells. Cells were independently cotransfected with (1) pCMVtetOprecore (1 μg) and pCDNA3 (3 μg); (2) pCMVtetO-precore (1 μg) and pCDNA3-tetR (3 μg); (3) pCMVtetOprecore (1 μg) and pCDNA3-tetR (3 μg) in the presence of tetracycline at 5 μg/mL after transfection.

We established the SSMC-7721-tetR cell line by stable transfection of 7721 cells with tetR-expressing

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

plasmid pCDNA3-tetR. The tetR-expressing cells were selected by growing cells in medium containing G418 at a concentration of 400 μg/mL. Fig.4 represents the result of transient transfection analysis of SSMC-7721-tetR cells with pCMVtetOprecore and presents precore protein expression in the absence or the presence of tetracycline.

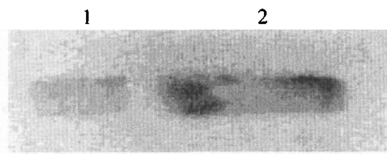

Fig. 4. Tetracycline induction HBV precore protein expression in SSMC-7721 cell line stably expressing tetR. After 24 h culturing, SSMC-7721-tetR cells were transfected with (1) pCMVtetOprecore (1 μg) in the absence of tetracycline; (2) pCMVtetOprecore (1 μg) in the presence of tetracycline.

3 Discussion

The novel tetracycline-regulated expression system is based on "tet-off" and "tet-on" tetracycline inducible expression system, and it demonstrates that the tetracycline repressor (tetR) alone, rather than tetR-mammalian cell transcription factor fusion-derivatives, can function as a potent-modulator to regulate gene expression in mammalian cells. In contrast to other inducible mammalian expression systems, this system does not use transcription activator fusion protein such as tTA (tetR-VP16) that can cause undersirable, pleiotrophic effects in many host cells[6,7].

In this study, we used quantitative FACS analysis and a novel tetracycline-regulated expression system to examine the induction of EGFP by tetracycline on a cell by cell basis in different cell lines. EGFP is less sensitive than luciferase, EGF and other report genes, but more readily assayed at the single cell level. For comparison of the expression of tetracycline regulated EGFP reporter gene, CHO K1 cells, HEK 293 cells and SSMC-7721 cells were used in our study. The FACS analysis demonstrated that the expression efficiency of pCMVtetOEGFP are the highest in CHO cells, moderate in 7721 cells and the lowest in HEK 293 cells. As for the level of tetracycline-mediated inducible gene regulation, different cells showed obviously different MFI values when the transfected cells were cultured in the absence or presence of tetracycline, which is related to the cell type and to the regulated expression efficiency. The induction of expression obtained upon presence of tetracycline was robust in CHO K1 cells, intermediate in SSMC-7721 cells but only modest in HEK 293 cells. Howe et al. have reported that HEK 293 cells do not provide a cellular environment in which the expression of a heterologous gene can be tightly controlled in the tetracycline-responsive manner[8], therefore, our results are in good agreement with Howe's.

Chronic hepatitis B is a common disease with a worldwide prevalence of more than 300 million carriers. HBV infection is a significant cause of liver disease and long-term sequelae of infection include chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Current established therapy involves treatment with alpha interferon but results in limited clinical success. Nucleic acid-based antiviral approaches for HBV include the use of antisense oligonucleotides and RNA, ribozymes, DNA-based therapeutic vaccines and dominant negative mutant core proteins that disrupt the life cycle of HBV[9]. An attractive antiviral strategy is based on the design and use of transdominant negative proteins that are able to interact and/or disrupt the function of their native counterparts. For example, cells overexpressing mutated forms of the HIV Rev or Tat proteins have been able to inhibit "wild-type" HIV replication[10~12]. In this regard, HBV precore proteins and core mutants were identified and functionally characterized as transdominant negative proteins that strikingly inhibit "wild-type" viral replication *in vitro* and *in vivo*[13,14]. The antiviral effect was principally due to interference by the precore proteins with "wild-type" nucleocapsid assembly. As a consequence of this phenomenon, the process of pregenomic RNA encapsidation was inhibited and viral DNA synthesis terminated. In this study, we have cloned HBV precore protein gene into tetracycline inducible plasmid pCMVtetO by PCR method and constructed plasmid pCMVtetOprecore. We showed that the precore protein gene expression can be regulated tightly by tetR in response to tetracycline when the cells were cotransfected with tetR-expressing plasmid in transient expression assays and the plasmid was transfected into a tetR-expressing cell line, SSMC-7721-tetR. Compared with other repression-based mammalian expression systems, the tetracycline-regulated expression system offers higher induced expression levels and lower basal level of expression under repressing conditions. What is more important is that this system does not use viral transactivating domains which are toxic to many mammalian cells. We utilized EGFP as the reporter to

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

study quantitatively regulated expression of tetracycline in several cell types. Moreover, our experiments demonstrated the expression of HBV precore protein gene can be accurately controlled in response to tetracycline in hepatocarcinoma cell lines. These results also provided useful data for the further studies in gene therapy for infectious HBV.

Acknowledgements The authors would like to thank Prof. Gao Guangxia for his kindly providing us plasmid TOPO-HBV and Prof. Huang Zuhu at Nanjing Medical College for his kindly providing us antibody against HBV core antigen. We also thank Liu Lin, and Xin Shuxia at the School of Basic Medical Sciences of Beijing University for performing FACS analysis. We are grateful to Ms. Liao Fulian for her technical support.

References

1 Gossen, M. et al. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. U S A, 1992, 89(12): 5547.

2 Gossen, M. et al. Transcriptional activation by tetracyclines in mammalian cells. Science, 1995, 268(5218): 1766.

3 Bujard, H. Controlling genes with tetracyclines. J. Gene. Med., 1999, 1(5): 372.

4 Wei, X. D. et al. Retrovirus vector expresses luciferase and TPO gene under the control of doxycycline in NIH cells. National Medical Journal of China, 2000, 80(10): 773.

5 Guidotti, L. G. et al. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. J. Virol., 1996, 70(10): 7056.

6 Yao, F. et al. Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum. Gene. Ther., 1998, 9(13): 1939.

7 Ogueta, S. B. et al. Design and in vitro characterization of a single regulatory module for efficient control of gene expression in both plasmid DNA and a self-inactivating lentiviral vector. Mol. Med., 2001, 7(8): 569.

8 Howe, J. R. et al. The responsiveness of a tetracycline-sensitive expression system differs in different cell lines. The Journal of Biological Chemistry, 1995, 270(23): 14168.

9 Wands, J. R. et al. Nucleic acid-based antiviral and gene therapy of chronic hepatitis B infection. J. Gastroenterol. Hepatol., 1997, 12(9~10): S354.

10 Gjerdrum, C. et al. Functional role of the HIV-1 Rev exon 1 encoded region in complex formation and trans-dominant inhibition. FEBS. Lett., 2001, 495(1~2): 106.

11 Fraisier, C. et al. Inhibition of Tat-mediated transactivation and HIV replication with Tat mutant and repressor domain fusion proteins. Gene Ther., 1998, 5(7): 946.

12 Bevec, D. et al. Inhibition of human immunodeficiency virus type 1 replication in human T cells by retroviral-mediated gene transfer of a dominant-negative Rev trans-activator. Proc. Natl. Acad. Sci. U S A, 1992, 89(20): 9870.

13 Lamberts, C. et al. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J. Virol., 1993, 67(7): 3756.

14 Wu, C. H. et al. Gene therapy applications in gastroenterology and hepatology. Can. J. Gastroenterol., 2000, 14(1): 57.

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

ZHU Xiaodong, et al. Controlled expression of enhanced green fluorescent protein and hepatitis B virus precore protein in mammalian cells

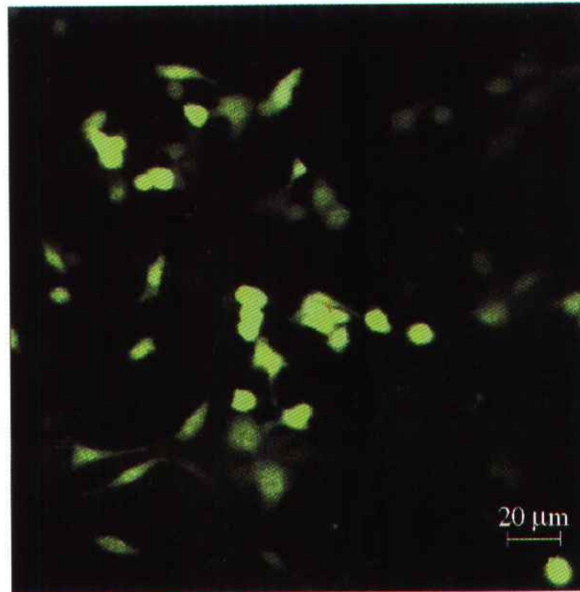

Plate 1. Hepatocellular carcinoma cell line SSMC 7721 transfected by pCMVtetOEGFP

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net