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The function activity comparison of eight promoters on glioma cells by gate way system

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ABSTRACT

With the development of modern molecular biology, clone and expresson technique is improved. For the study the mechanism of GDNF promoting the proliferation of glioma cells, many genes need to knock down or over-expression. So we need an effective protein expression system. In this study we use Gateway Technology, which is easy for us to construct a expression vector.. Promoter is a very important element for a protein expression, because some promoters maybe not promote the expression of the protein in glioma cells, some promoters maybe too strong, which will kill the cell after transformation. So we systematicly compare nine promoters which we have. After comparison, we found the CMV, SV40, PGK promoters are all work well in glioma cells, and the pgk is the best one. BetaActin dosen't work well, which will cause glioma cells to die.

KEYWORDS

Activity comparison; Promoters; Glioma; Gatewaysystem.

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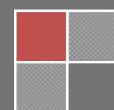


INTRODUCTION

Cloning and expression technology is the core and basic techniques of modern molecular biology. Scientists have already set up a systematic cloning and expression technique for prokaryotic and eukaryotic cells. Prokaryotic expression system is simple, but eukaryotic expression is complicated. No matter what kind of expression system, promoter always is one of the most important factors. Several viral promoter have been utilized to drive gene and expression, including sequences derived from cytomegalovirus (CMV), Simian virus 40(SV40)[1]. In this study, I chose eight promoters, which are used often in current molecular biology research. And test them on two glioma cells, which will be used often in our study teem for the current research.

Gateway Technology is an innovative and highly efficient method for protein expression and functional analysis. Based on lambda site-specifc recombination[2], MultiSite Gateway technology enables easy, accurate and efficient assembly of multiple DNA fragments in the order and orientation into a single vector. It offers us the most flexible system for comprehensive gene analysis, protein expression, and functional analysis. Six types of Gateway recombination signals, att1, att2, att3, att4, att5 and att6only between the matched att signals, for example attL3 to attR3 but not attL3 to attR5, and so on. The Gateway system as a tool for high-throughput production of Expression clones by preferential recombination between specific att signals in one reaction. Examples of high-throughput construction of the recombinant plasmid containing a fusion structure of three or four DNA molecules are att6, can be recombined specifically and effectively[3].

Two recombination reactions, LR and BP, constitute the Gateway cloning technology. The LR reaction is the in vitro version of the phage lambda excision reaction. Mediated by LR clonase through attL and attR sites, transfer of the target DNA fragment in the Entry clone to a Destination vector generates an Expression clone.

The BP reaction transfers the DNA fragment of the Expression clone or the attB-flanked PCR product to a Donor vector through attB and attP by BP clonase, creating an Entry clone. In this study, we take advantage of a highly effcient, overnight recombination reaction to transfer fragments from one vector to the next. It is easy for us to shuttle one part DNA to a Vector.

Promoters EF1a and Ubiquitin are from Human, Copia and Actin5C are from drosophila, Pgk is from Mouse, CMV and SV40 are from virus. We use powerful multiple gateway system to construct expression vectors for nine promoters. For detection we use EGFP as a strong marker. By fluorescence microscope and Flow cytometry system to compare the amount of GFP expression. Usually the promoter works better in the cells of organism which the promoter is from. Maybe for one species, one cell type of same species, even same cell type but different phage of the cells development, the promoter sequence, the mechanism, and the process modification of gene after transcription, so the GFP shows different expression situation[5].

MATERIALS AND METHODS

Overview of the two gene expression clone construction

Construction of two cDNA-tandem expression clones. Two Entry clones and a Destination vector, respectively. Two entry clones are got by BP reaction. Generation of two cDNA-tandem expression clones by LR recombination of Pup (pENTR-1) and pDown (pENTR-2) with a pDEST (-R4R2) harboring a CmR–ccdB cassette. Two pairs of attL–attR signals recombine specifically with each other[6].

PCR of Promoters and fluorescent proteins genes

The size and the percentage of GC between different PCR products are very different, so we have to optimize the PCR condition. The output variables were the efficiency (yield) and specificity of the PCR, which are the two most important characteristics of PCR. The obtained ANN model was characterized by both descriptive and predictive ability ($R_d^2 = 0.993$ and $R_p^2 = 0.987$). The correlation coefficients indicated that the proposed method could provide a more accurate optimization than the existing methods in literatures PCR **103**[4]. Based on the reliable predictive ability of ANN, the effects of each influencing parameter on PCR efficiency and specificity were predicted.

TABLE 1 : The original templates of promoters

DNA	species	Size	the original plasmid	company
UBC	Human	1177	pBL1628	Lahnlab
COPIA	Drosophila	1600	pUC-HygroMT	DGRC
ACT5C	Drosophila	300	pUC-act-DHFR	DGRC
PGK	Mouse	511	pcDNA6.2/EmGFP-Bsd/V5-Dest	Invitrogen
EF1A	Human	1264	pTracer-EF alpha	Invitrogen
CMV	Virus	589	pCMV-tdTomato	Clonetech
TRE	synthetic	434	pTRE2hyg	Clonetech
CAGG	Chicken	1720	pBL1653	Lahnlab
SV40	Virus	344	pTracer-SV40	Invitrogen
GFP	marine jellyfish	720	pIRESneo-GFP-alpha	Addgene

To get promoter PCR products flanked by two different attB site. The recombinant plasmids containing promoers or the fluorescent protein EGFP were used as templates for PCR.

Entry clones

Then entry clones (pENTR) containing the promoters or fluorescent protein EGFP-ORFs were constructed as follows. For preparing the DNA fragment with attB signals, for example, attBx-EF1a-(ORF)-attBy, a pair optional containing attB sequence. primers and a Vectors harboring the c DNA were used as template to amplify the ORF fragments by PCR. Amplification reactions were assembled using a mixture of forward primers (Fw) (Bx-EF1a and a reverse primer (Rv) By-EF1a. In preparing the N-terminal fusion consisting of attB-flanked ORF fragments, the Bx-EGFP-Fw primer and By-EGFP-Rv primer were used to amplify the attBx-EGFP (ORF)-attBy. The attB-flanked PCR product s were recombined with the donor vectors containing the corresponding attP signals in the BP reaction to generate the entry clones. The entry colones were confirmed by sequence with ABI PRISM 3700[7].

In vitro recombination reactions

BP and LR reactions were performed using BP Clonase™ Enzyme Mix (Invitrogen Corp.) and LR Clonase™ Enzyme Mix (Invitrogen Corp.) according to the supplier's instructions and as reported previously[8]. Although LR Clonase™ Plus Enzyme Mix (Invitrogen Corp.) is recommended for the multi-site gateway LR reactions,. Using the PureLink™ HiPure Plasmid Midiprep Kit (Catalog no. K2100-04) prepare plasmid DNA. To get high reaction efficiency we use the following formula to calculate exactly.

Convert fmoles to nanograms (ng)[9]
Ng=(xfmol) (N)(660fg/fmol)(1ng/106fg)
where N is the size of the DNA in base pairs, and x is the number of fmoles
We recommend 10 fmol of each entry
clone and 20 fmol of DEST vector per 10 µl reaction.

Add the following components to a 1.5-ml microcentrifuge tube at room temperature and mix. Use this reaction mixture in the Procedure, it was difficult to obtain a positive clone if use more or less either plasmids. The DNA amount that we used in LR reaction were calculate as TABLE 3.

TABLE 3 : The reaction of LR

pUP	Dest vector (bp)	LR pUP-amount (ng)	pUP size (bp)
pUP-UBC	4820	15.91	4342
pUP-COPIA	5243	17.30	4765
pUP-ACT5C	3943	13.01	3465
pUP-PGK	4154	13.71	3676
pUP-EF1A	4907	16.19	4429
pUP-CMV	4232	13.97	3754
pUP-CAGG	5363	17.70	4885
pUP-SV40	3987	13.16	3505
pDown-GFP	4359	14.38	-------
pDEST-R4R2	4107	27.10	-------

The total 5µl reaction system. Entry clones 5 fmoles, destination vector 10 fmoles. 1X TE buffer, pH 4.0 to 4 µl.

LR Reaction Procedure[9]
a. Remove LR Clonase™ II Plus enzyme mix from freezer and thaw on ice for about 2 minutes. Vortex the enzyme mix briefly twice (2 seconds each).
b. To each MultiSite or MultiSite Pro LR reaction mixture, add 2 µl of LR
Clonase™ II Plus and mix well by vortexing briefly twice. Microcentrifuge briefly.
c. Return enzyme mix to freezer immediately after use. The enzyme mix can be stored at -20°C for up to 6 months or at -80°C for long-term storage.
d. Incubate recombination reaction at 25°C for 16 hours.
e. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Bacterial host and transformation

For construction of donor vectors and destination vectors containing the ccdB gene[12], Library Efficiency® DB3.1™ or One Shot® ccdB Survival 2 T1R Competent Cells (Invitrogen Corp.) were used. For construction of other vectors and clones without a ccdB gene, Max Efficiency Top10 Competent Cells or One Shot® Stbl3™ Chemically

Competent Cells (Invitrogen Corp.) were used. In order to reduce the double transformed colonies (containing both entry and expression clones or both donor and destination vectors) or a co-integrant intermediate product (fusion constructs of an entry clone and a destination vector or an expression clone and a donor vector resulting from recombination at only one of two pairs of att sites), secondary screening by inoculating the obtained colonies onto two LB-agar plates, the first one containing the same antibiotics (ampicillin or kanamycin) with which they were formed and the second one containing the antibiotics other than the first one. The clones, which formed colonies on both plates are false clones containing double transformants or the intermediate products, thus these were omitted. The plasmid DNA of each clone was isolated using Quantum Prep Mini Kit (Bio-Rad Laboratories) and the size and digestion pattern was confirmed by restriction enzyme digestion followed by agarose gel electrophoresis. Finally sequence to testify them. The clones with the correct size and pattern were stored as true positive clone and used for further experiments.

Transfecion to glioma cells human U-373MG and rat C6 for expression assay

One constructed, the multi- cDNA tandem expression clones were transfected into two candidate cells. All cell lines were maintained in media supplemented with 10% fetal bovine serum (Invitrogen Corp.), in the low glucose DMEM (GIBCO invitrogen).

The transfections were carried out using lipo from Invitrogen.

Observation of a fluorescence microscope

Three days later the cells were observed with a fluorescence microscope, Olympus Research microscope IX51S8F-3, The IX51 is designed for many routine and research applications including advanced fluorescence techniques. The slim frame enables easy attachment of multiple accessories for cell culture and imaging applications. Excellent optical performance and mechanical quality have been combined into a microscope system of outstanding value.

RESULTS

Construction of pUP, pDown and dest expression clones

For constructing this type of expression clone, four different types of att signals were used. By employing stepwise strategy, we are successful to get eight expression vectors.

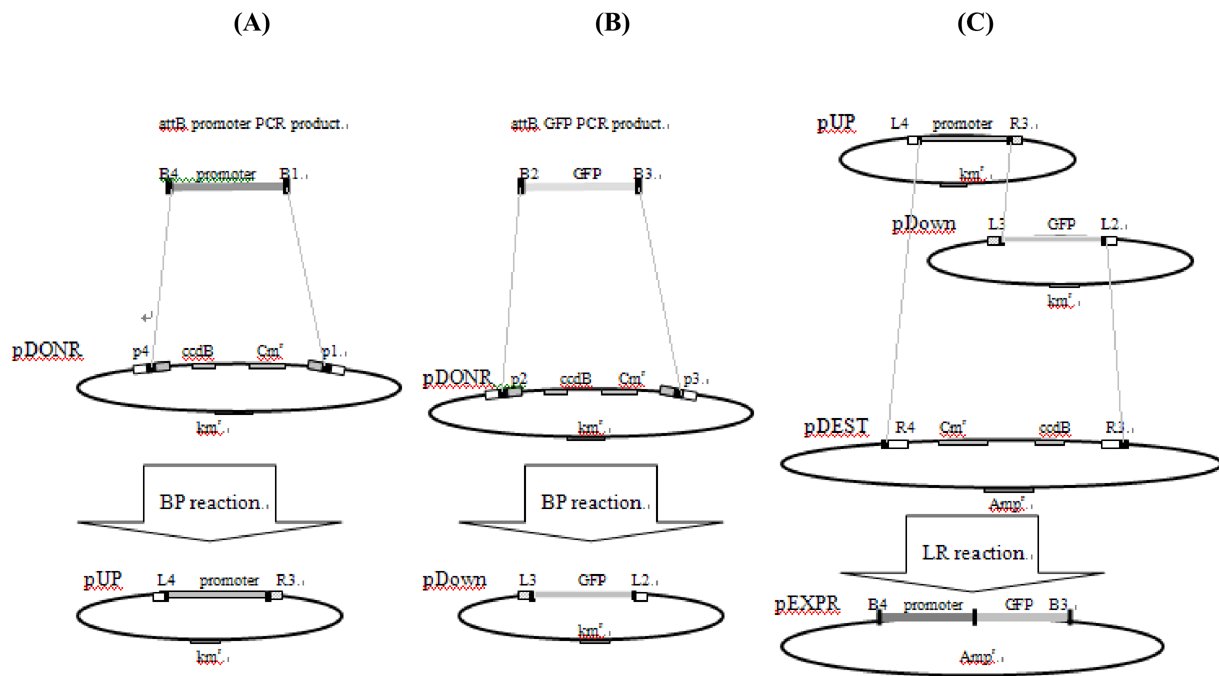
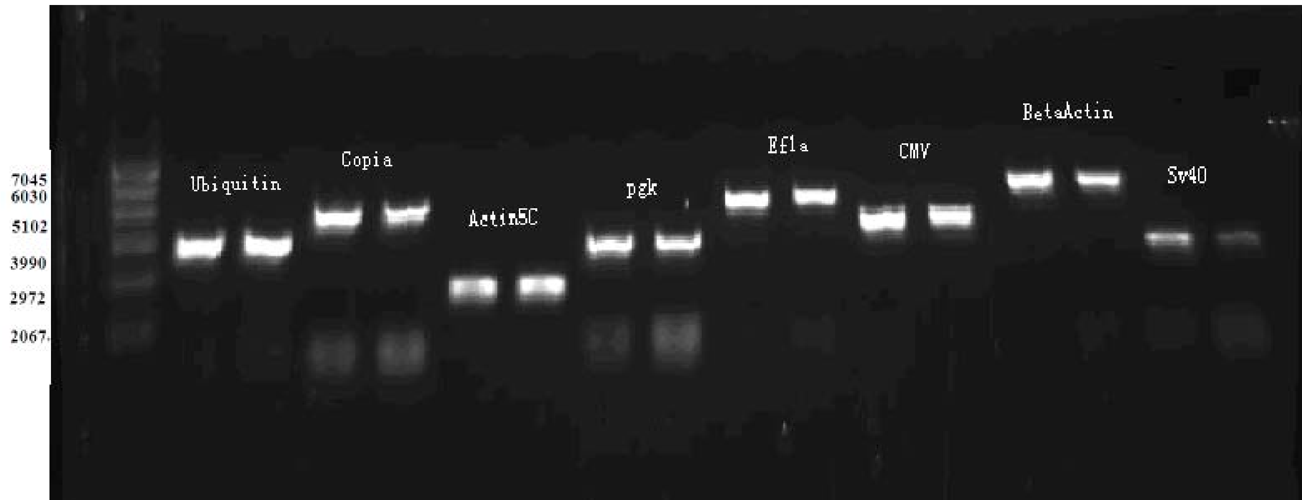


Figure 1.A : pUp-promoter (pENTRY 1); B : pDown-EGFP (pENTRY 2); C : Two entery clones and a destination vector harboring site of the CmR–ccdB cassette in one LR reaction.

LR reaction

Construction of a three cDNA-tandem expression clone using a modular destination vector and a two cDNA-tandem entry clone by LR recombination. The competent cells, Stbl3 were used for pEXPR. The other conditions and representations are as in TABLE 2.

A B C D E F G H I



Figture 2 : From left, the first lane is lader. Second and third are BetaActin drive EGFP; Fourth and Fifth are promoter copia drive EGFP; Six and Seventh lanes are promoter Actin5C drive EGFP; Eighth and Ninth are promoter pgk drive EGFP; Tenth and Eleventh lanes are EF1a drive EGFP; Twelfth and thirteenth are CMV drive EGFP; Fourteenth and fifteenth are inducible promoter tre drive EGFP; sixteenth and seventeenth are promoter BetaActin drive EGFP; Eighteenth and nineteenth are promoter Sv40 drive EGFP. Gel pictures are test the productes are right. Then sequence them to make sure we get correct LR reaction products.

Fluorescence microscope

An average amount of 1ug DNA was transfected per well of six well plate. The GFP expression image on C6 glioma cells:

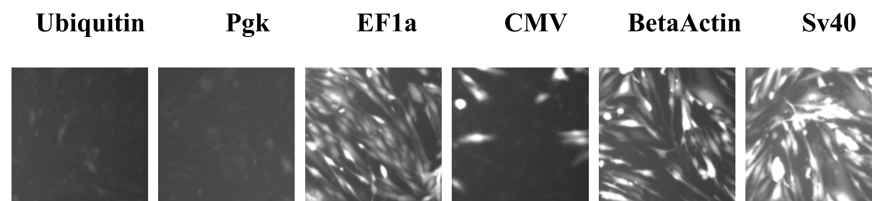


Figure 3 : The GFP expression in C6 glioma cells transfected, from left, with pDEST-Ubiquitin-GFP, pDEST-pgk-GFP, pDEST -EF1a-GFP, pDEST -CMV-GFP, pDEST -BetaActin-GFP, pDEST -Sv40-GFP.

DISCUSSION

The multi-site gateway system employed in this report features rapid and convenient cloning of multiple cDNAs. By using six types of att signals, such as att1, att2, att3, att4, In the present studies, we investigated the effects of different promoters on the levels of expression of genes of interest rather than reporter gene constructs. We chose six ubiquitous promoters, which are common used on the mammalian cell's regular expression. We create nine expression clones using nine different promoters driving EGFP fluorescence protein marker. It is easy and make sure those promoters driving EGFP are all at exactly the same site at a same vector. The expression of GFP protein fluorescent marker is becoming a research tool of growing importance for functional genomics. Here we compare the promoters activities by measuring the GFP expression signals.

Plasmid vectors are important, convenient, and thus frequently used tool for various molecular biological applications. And the right promoter is very important for the vector construction. We found that the CMV and pgk promoters are the best two for glioma cells. For the promoter Ubiqutine, the fluorescence is strong, but the cell type doesn't look well. get to the strongest. But in different cells, the GFP fluorescence are different. In the C6 cells, The fluorescence is we demonstrated a 2-fold increase for EF1a promoter. For the rest promoters, the luciferase expression is between them.

Usually the promoter works better in the cells of organism which the promoter is from. Maybe for one species, one cell type of same species, even same cell type but different phage of the cells development, the promoter sequence, the mechanism, or some factors affect the transcription, so the GFP shows different expression level[10].

Our data may be useful to others in choosing the appropriate promoters for gene transfer applications that require minimum to high levels of expression in glioma cell lines.

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