Spring 5-1-2005

Developing a Constitutively Active Aryl Hydrocarbon Receptor in the Mouse Heart

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INTRODUCTION

According to recent statistics published by the American Heart Association, cardiovascular disease claims more than 930,000 lives each year. That is more than the next five leading causes of death combined. Major risk factors for developing cardiovascular complications include obesity, dyslipidemia, diabetes, age greater than 60, male gender, family history, and smoking[3]. The importance of smoking cessation is matched only by the gravity of the risk smoking itself presents. An estimated 20 to 30% increase in myocardial infarctions or ischemic heart disease has been exhibited even in nonsmokers exposed to substantial amounts of environmental tobacco smoke[4]. The lipophilic nature of the various polycyclic aromatic hydrocarbons (PAHs) found in tobacco smoke expedites their access into a cell, thereby intensifying their harmful effect.

A well-conserved protein known as the aryl hydrocarbon receptor (AHR) mediates the response to such PAHs. The AHR is a ligand-dependent, bHLH-PAS transcription factor found throughout the body, including the heart, liver, lungs, and thymus[5]. Upon activation, it regulates the expression of its target genes by binding to xenobiotic response elements (XREs) on DNA. One important group of target genes is the xenobiotic-metabolizing enzymes that are up-regulated as an adaptive response to many environmental stressors. These enzymes shorten the half-life of the insulting stressor, thereby limiting its harmful effect. The bHLH (basic, helix-loop-helix) domain participates in homotypic dimerization interactions that position the basic region in place for contact with regulatory elements found in DNA. The PAS (Per-Arnt-Sim) domain functions as a surface

for the following: homotypic interactions with other PAS proteins, heterotypic interactions with chaperones like Hsp90, and interactions with activating ligands[6].

The unbound AHR is found in the cytoplasm of a cell, clustered with a dimer of Hsp90 and additional cellular chaperones such as ARA9[5-7]. This complex, especially the binding with Hsp90, is thought to appropriately align a site on the AHR necessary for ligand binding. Subsequent binding of a ligand exposes a nuclear localization signal on the receptor, thereby promoting translocation of the complex into the nucleus. Upon entering the nucleus, the AHR dissociates itself from its chaperone proteins and forms a heterodimer with another bHLH-PAS protein, ARNT. ARNT is responsible for the direction of the ligand-bound AHR to specific enhancer elements on the DNA. The enhancer element to which the AHR/ARNT heterodimer binds is called a xenobiotic response element (XRE). The core XRE sequence is TNGCGTG, in which AHR binds TNGC and ARNT binds GTG. Binding of the AHR/ARNT complex to the XREs allows for enhanced transcription of specific target genes. Many of these genes are drug-metabolizing enzymes and include phase one enzymes (i.e. CYP1A1 and CYP1A2) and phase two enzymes (i.e. NADPH-quinone oxidoreductase and GST-1a). An additional protein known as the AHR repressor (AHRR) is up-regulated, and presumed to be involved in a negative feedback loop by competing with AHR for ARNT dimerization[8].

The known xenobiotic ligands for the AHR include PAHs (i.e. 3-methylcholanthrene) and halogenated aromatic compounds (i.e. 2,3,7,8-tetracholorodibenzo-p-dioxin or dioxin)[6;9;10]. The greatest sources for human

exposure stem from large-scale industrial processes, including the incomplete combustion of plastics and pulp and paper production. More common sources include cigarette smoke, exhaust fumes, diesel engines, cooked meat and fish, dairy products, and mother's milk. Dioxin is the most studied xenobiotic ligand for the AHR. This halogenated aromatic compound binds solely to the AHR, is slowly metabolized by the body, and is thought to bind to the receptor with an even greater affinity than other PAHs[6;9;10].

Toxicological studies in which entire organisms were exposed to vast amounts of xenobiotics have confirmed that overactivation of the AHR leads to many systemic effects, including acute lethality, chloracne, immune suppression with thymic atrophy, and alterations in growth factors and cytokines[6]. Overexposure has also been linked to cancer and cardiovascular disease[11-14]. Cardiotoxicity due to TCDD overexposure was illustrated by a decrease in both heart rate and blood pressure[6].

Compared to the xenobiotic ligands, very little is known about the endogenous ligands for the AHR. This group of comparatively short-lived molecules is suspected to include 7-ketocholesterol[15] and bilirubin[16;17]. AHR knockout mouse strains have been developed to explore what role the AHR plays in normal physiology. Although it was found that these mice were no longer susceptible to the effects of dioxin exposure, other serious physiologic problems arose. The knockout mice exhibited persistent fetal vasculature and liver morphology, compromised immune systems, and both structural and functional cardiac abnormalities[18]. Specifically in the mouse hearts, an observed increase in

the size of cardiac myocytes was consistent with hypertrophy [19]. Although the molecular mechanism remains unclear, the results of these studies confirm that the AHR is necessary for growth, survival, and reproduction in the normal physiology of the mouse.

Although the overactivation and knockout studies have provided great insight into the effects of extreme degrees of AHR activity, many questions are left unanswered by their results. One caveat of whole animal dosing experiments is their failure to target a specific cellular population, thereby blurring the line between cell autonomous and non-autonomous effects. My project aims to solve this problem by constructing a conditional transgene that drives constitutive activation of the AHR only in the cardiomyocyte, or any other desired specific cellular population. This approach utilizes the Green Fluorescent Protein (GFP) along with a cre-lox P system for detectable, site-specific activation of the AHR protein. The following paper describes the design strategy and construction of this type of AHR transgene.

MATERIALS and METHODS

Antibiotic Selection

The antibiotic Zeocin was added to LB agar plates for selection of colonies containing the pDRIVE vector. Ampicillin was used as a selectable marker for the remainder of the constructed plasmids.

DNA isolation and purification

QIAGEN Miniprep kits were used to isolate purified plasmid DNA from *E. coli* cells. The procedure is based on the alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt.

Pelleted cells from the bacterial culture were resuspended in Buffer P1 (50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). The cells were then lysed under alkaline conditions upon addition of Buffer P2 (200 mM NaOH and 1% SDS [w/v]). The lysate was subsequently neutralized with Buffer N3 (proprietary mixture of guanidine hydrochloride, isopropanol, and other agents), which causes the genomic DNA, proteins, carbohydrates, and lipids to precipitate while re-naturing the plasmid DNA. After separation of the precipitate from DNA, the plasmid DNA was exposed to the silica-gel membrane and bound due to the high salt conditions. Buffer PB (proprietary mixture of guanidinium chloride, propan-2-ol, and other agents) and Buffer PE (proprietary mixture of ethanol and other agents) were used to wash the silica-gel membrane. The purified DNA was then collected by elution with Buffer EB (10 mM Tris·Cl, pH 8.5).

Ethanol Precipitation

An ethanol precipitation was performed to increase the concentration of the DNA sample. After the volume of the DNA sample was measured, the salt concentration was adjusted by adding 1/10 volume of 3 M sodium acetate, pH 5.2. Next, 2.5 volumes of 100% ethanol were added and the mixture was placed on ice for a minimum of 20 minutes. The DNA was pelleted by centrifugation, separated from the supernatant, and then washed with 1 ml of 70% ethanol. The sample was again centrifuged, separated from the supernatant, and allowed to air dry for 30 minutes. The pellet was then resuspended in the appropriate volume of TE (10 mM Tris-HCl, pH 8, with 1 mM EDTA).

Gel Electrophoresis

To determine the size and concentration of DNA fragments, DNA samples were loaded on 0.7%, 1.0%, or 2% agarose gels. For size determination, samples were run against a 1kb ladder (Invitrogen). For concentration determination, samples were run against either a high mass or low-mass ladder (Invitrogen), depending on the size of the fragment.

The concentration of agarose in the gel was dictated by the size of the DNA fragments being separated. The agarose gels were prepared by dissolving the appropriate amount of agarose in 300 ml of 1X TAE buffer (40mM Tris; 20mM acetic acid; 1mM EDTA). 15µl of 10mg/ml ethidium bromide was added for visualization under UV light. Approximately 50ml was poured for each gel.

Sources of plasmid components

Oligos inserted in ligation reaction with pBluescript II SK plasmid (pSK) were ordered from Invitrogen. The CAG promoter was isolated from the pDRIVE-CAG vector (InvivoGen). The GFP was isolated from the pd2EGFP-1 vector (BD Biosciences). The mouse β globin terminator and poly A addition sequence (gDEF) was obtained from Dr. Falck-Pedersen[20].

Spectrophotometric assay

100 µl DNA samples were loaded into a 100 µL microcuvette in 1:25, 1:50, or 1:100 dilutions with TE (1 M Tris-HCl, pH 8, with 100 mM EDTA). The assays were performed using a BioRad Spectrophotometer and the absorbency readings at 260λ and 280λ were recorded. Nucleic acid concentrations of dsDNA were determined by multiplying the 260λ absorbance reading by a mass/absorption conversion factor and by the dilution factor. The mass/absorption conversion factor used for dsDNA was 50.0 µg/ml.

RESULTS

Design of conditionally activated DNA construct

The final conditional transgene expression construct should contain a ubiquitous promoter, a translational insulator flanked by two lox P sites, the coding region of the target gene, and a poly A addition signal (Figure 2, A).The CAG promoter is a very strong and ubiquitous promoter that will be used in my construct. It has been shown to drive expression in all tissues of transgenic mice with the exception of erythrocytes and hair[21]. This composite promoter combines the human cytomegalovirus immediate-early enhancer, a modified chicken beta-actin promoter, and a 5' untranslated region[22].

The GFP marker will function as an insulator of translation as well as a selectable marker for those cells expressing the target genes driven by the CAG promoter. Important components include the EGFP coding region, a PEST sequence, and SV40 Poly A region. The PEST sequence destabilizes the protein, thereby reducing its half-life and preventing the toxic effects of an overabundance of GFP. The translational stop site (TGA) prevents the translation of the target gene until the GFP is acted upon by a cre-lox P gene switch approach. In the presence of the cre-recombinase, the DNA encoding GFP between the lox P sites will be looped out, allowing transcription and translation of the target gene (Figure 2, B). A cell-specific promoter can be used to drive the Cre, thereby enabling expression of the target gene in a specific cell population.

The mouse β−globin transcriptional terminator and poly A addition signal, gDEF, will be included at the 3'-end of the target gene coding region to provide for mRNA stability [20].

Construction of pBVD1

A 98 base pair oligo sequence was designed to include *Hin*dIII overhangs, two lox P sites, and additional restriction sites for subsequent cloning steps. The BVD1 plasmid results from the insertion of this oligo sequence into the pBluescript II SK plasmid (Figure 3, A and B). The pSK was first digested and linearized with *Hin*dIII. The complementary oligos were annealed to each other and then ligated to the resulting overhang sites on the pSK left by the *Hin*dIII digestion. A size shift of approximately 100 base pairs on a 2% agarose gel and the appearance of the *Age*I and *Afl*II restriction sites verified insertion of the oligo sequence. Proper orientation was confirmed by DNA sequencing.

Construction of pBVD2

The CAG promoter will be used to drive expression of the target genes in all tissues of transgenic mice with the exception of erythrocytes and hair. The BVD2 plasmid results from the insertion of the CAG promoter sequence into pBVD1 (Figure 4, A and B). The 1.6 kb CAG promoter sequence was first excised from the 6353 bp pDRIVE-CAG plasmid by *Pst*I and *Nco*I. The 5'

overhang left by the *Nco*I digest was blunted using T4 DNA polymerase. BVD1 was prepared for ligation by digestion with *Pst*I and *Eco*RV.

Construction of pBVD3

The GFP marker will function as an insulator of translation as well as a selectable marker for those cells expressing the target genes driven by the CAG promoter. The BVD3 plasmid results from the insertion of the GFP sequence into BVD2 (figure 5, A and B). The 1.2 kb GFP sequence was first excised from the 4.3 kb pd2EGFP-1 plasmid by *Bsh*T1 (an isoschizimer of *Age*I) and *Bsp*T1 (an isoschizimer of *Afl*II). BVD2 was prepared for ligation by digestion with *Bsh*T1 (*Age*I) and *Bsp*T1 (*Afl*II).

Construction of pBVD4

The gDEF sequence will function as a strong transcriptional terminator and poly A addition signal[20]. The BVD4 plasmid results from the insertion of the gDEF sequence into BVD3 (figure 6, A and B). The 1.5 kb gDEF sequence was first excised from the pMK1 plasmid by *Sal*I and *Not*I. The 5' overhang left by the *Not*I digest was blunted using T4 DNA polymerase. BVD 3 was prepared for ligation by digestion with *Sal*I and *Acc*65 I (an isoschizimer of *Kpn*I). The 5' overhang left by the *Acc*65I digest was blunted using T4 DNA polymerase.

DISCUSSION

Although time restraints have prevented me from inserting the AHR sequence into the pBVD4 plasmid, the stage has been set to explore the effects of conditional, constitutive activation of the AHR specifically in the cardiomyocyte of the mouse. Once the construct is complete and a stable transgenic line is produced, the cre-lox P system and GFP protein will allow those who continue my project to track AHR expression under a UV fluorescent microscope. I expect chronic, supraphysiologic activation of the AHR signaling pathway in the cardiomyocyte to be sufficient to perturb normal cardiac homeostasis. An additional exciting and valuable element of my construct lies in the research possibilities it has allowed for others. With my construct, essentially anyone with the appropriate cell-specific promoter driving cre-recombinase can conditionally and detectably activate any gene of interest in a specific cellular population.

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APPENDIX A

Figure 1: AHR mechanism upon xenobiotic activation

Unbound AHR is found in the cytoplasm of the cell, clustered with Hsp90 and other chaperone proteins. Upon ligand activation, a nuclear localization signal on the receptor is exposed, thereby promoting translocation of the complex into the nucleus. The AHR dissociates itself from its chaperone proteins upon entering the nucleus, and forms a heterodimer with ARNT. Binding of the AHR/ARNT complex to the XREs allows for enhanced transcription of specific target genes, including CYP1A1 and AHRR.

Figure 2: Effect of cre-recombinase on generic conditional transgene

A.

Schematic diagram of generic conditional transgene before cre-recombinase. The insulator, flanked by two lox P sites, contains a stop site that prevents the transcription of the gene of interest.

B.

Schematic diagram of generic conditional transgene after cre-recombinase. The cre-recombinase has acted at the lox P sites to excise the insulator, thereby allowing the transcription and translation of the gene of interest.

Figure 3: Insertion of oligo sequence into pSK

A.

Oligo sequence designed for insertion into pSK. Two identical 34 base pair lox P sequences (red) flank the *Age*I and *Afl*II restriction sites (red). The *Hin*dIII overhangs on each side of the oligo sequence were designed to complement the *Hin*dIII overhangs on the pSK during the ligation reaction.

B.

Plasmid map of pBVD1. Important sites on the oligo insert are labeled in red. *Pst*I and *Eco*RV sites will be used for subsequent insertion of CAG promoter.

Figure 4: Insertion of CAG promoter into pBVD1

A.

Schematic diagram of strong, hybrid CAG promoter. This composite promoter combines the human cytomegalovirus immediate-early enhancer (dashed), a modified chicken beta-actin promoter (dotted), and a 5' untranslated region. *Pst*I and *Nco*I sites were used for the ligation with *Pst*I and *Eco*RV sites on the pBVD1.

B.

Plasmid map of pBVD2. The CAG promoter and its important sites are labeled in blue. *Age*I and *Afl*II sites will be used for subsequent insertion of GFP.

Figure 5: Insertion of GFP into pBVD2

A.

Schematic diagram of GFP. Important components include the EGFP coding region (solid), PEST sequence (dashed), and SV40 Poly A addition signal (crossed). Translational start and stop sites are indicated. *Age*I and *Afl*II sites were used for the ligation with *Age*I and *Afl*II sites on the pBVD2.

B.

Plasmid map of pBVD3. The GFP and its important sites are labeled in green. *Sal*I and *Kpn*I sites will be used for subsequent insertion of gDEF.

Figure 6: Insertion of gDEF into pBVD3

A.

Schematic diagram of gDEF. The sequence includes a strong, mouse β-globin transcription terminator as well as a poly A addition signal for message stability.

B.

Plasmid map of pBVD4. The gDEF is labeled in purple. The restriction sites between the lox P and gDEF can be used for subsequent insertion of the AHR, or any other target gene.

APPENDIX B

Figure 1.

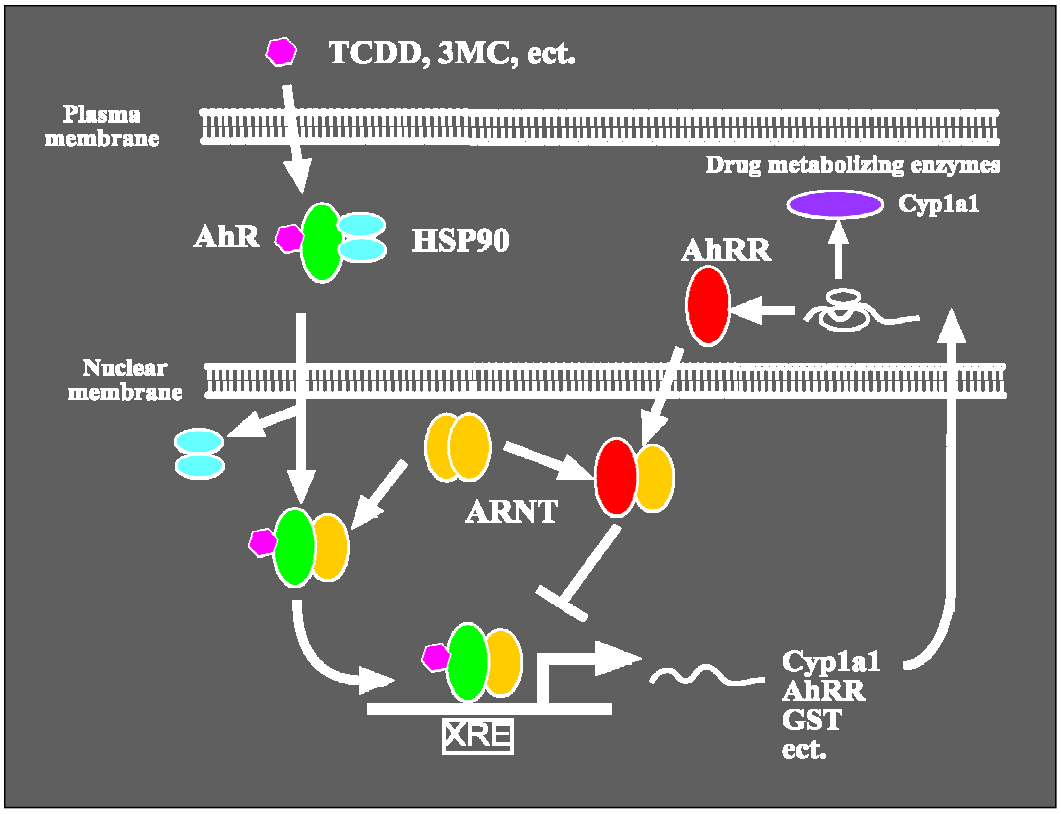


Figure 2.

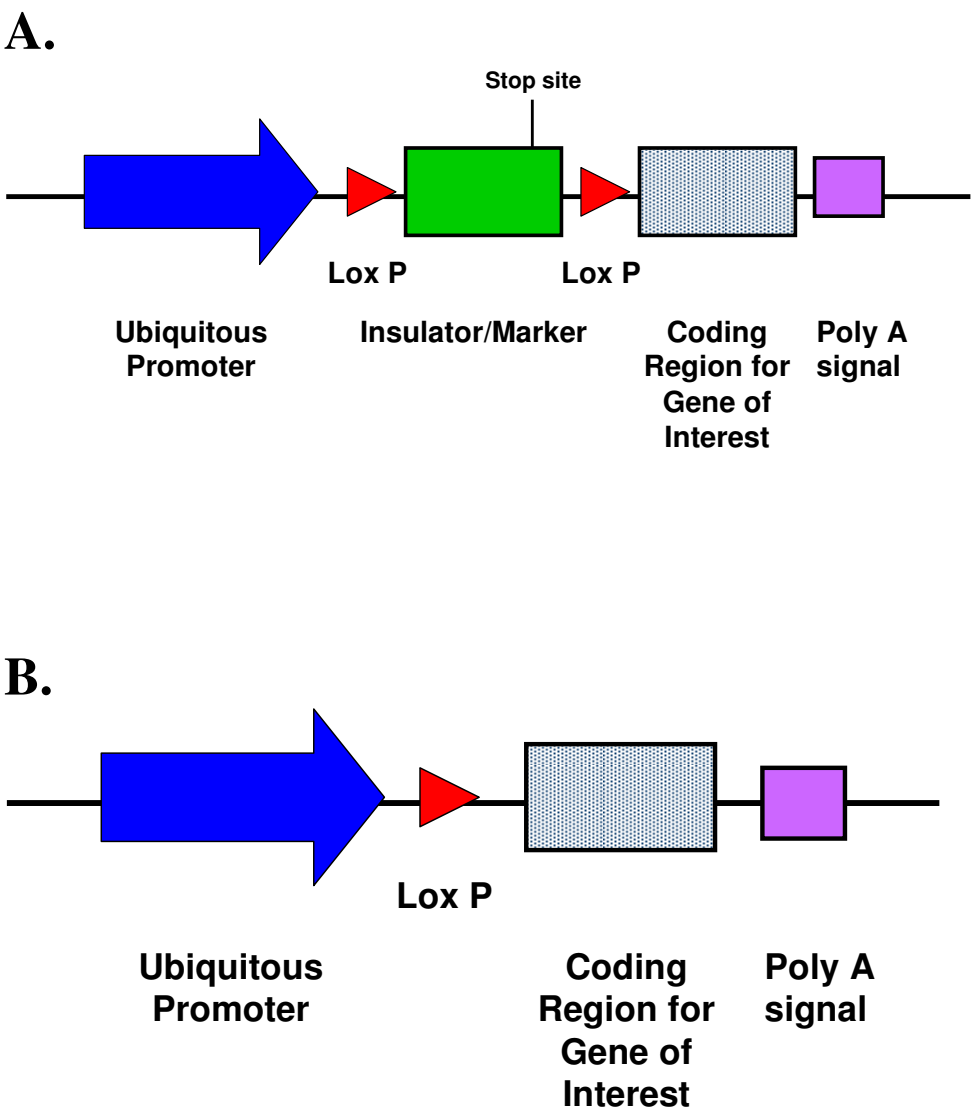

A.
Stop site
Lox P
Lox P
Ubiquitous Promoter
Insulator/Marker
Coding Region for Gene of Interest
Poly A signal
B.
Lox P
Ubiquitous Promoter
Coding Region for Gene of Interest
Poly A signal

Figure 3.

A.


Hind III overhang
Age I
Afl II
Hind III overhang
LOX P
LOX P
5' AGCTTGATAACTTCGTATAGCATACATTATACGAAGTTATACCGGTACCCAGCTGAAGCTTAAGATAACTTCGTATAGCATACATTATACGAAGTTAT 3'
3'ACTATTGAAGCATATCGTATGTAATATGCTTCAATATGGCCATGGGTCGACTTCGAATTCTATTGAAGCATATCGTATGTAATATGCTTCAATATCGA 5'

B.

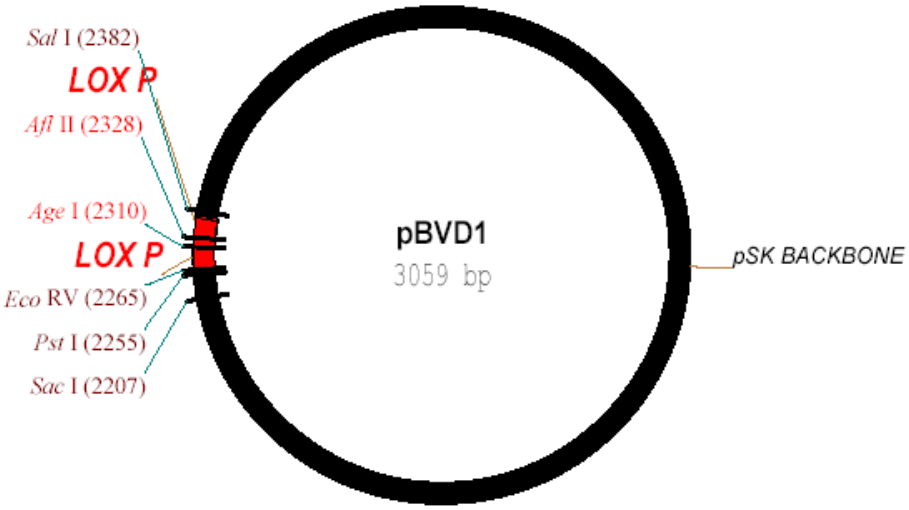
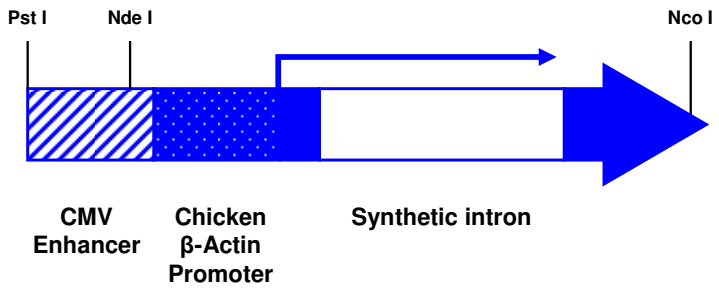

Sal I (2382)
LOX P
Afl II (2328)
Age I (2310)
LOX P
Eco RV (2265)
Pst I (2255)
Sac I (2207)
pBVD1
3059 bp
pSK BACKBONE

Figure 4.

A.


Pst I
Nde I
Nco I
CMV Enhancer
Chicken β-Actin Promoter
Synthetic intron

B.

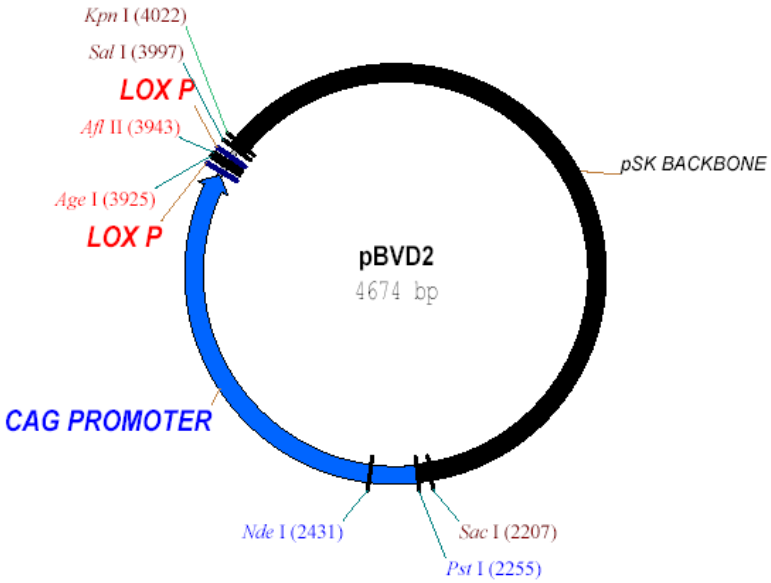

Kpn I (4022)
Sal I (3997)
LOX P
Afl II (3943)
Age I (3925)
LOX P
pSK BACKBONE
pBVD2
4674 bp
CAG PROMOTER
Nde I (2431)
Sac I (2207)
Pst I (2255)

Figure 5.

A.


Age I
ATG (start)
TGA (stop)
AfI II
EGFP
PEST
SV40
Poly A

B.

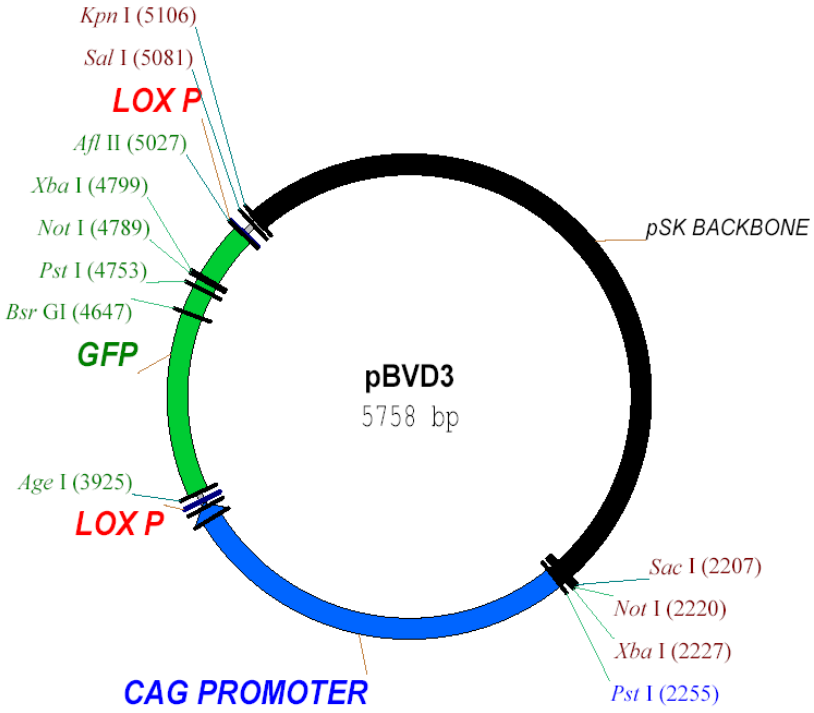
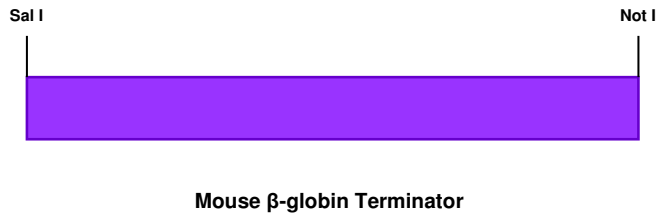
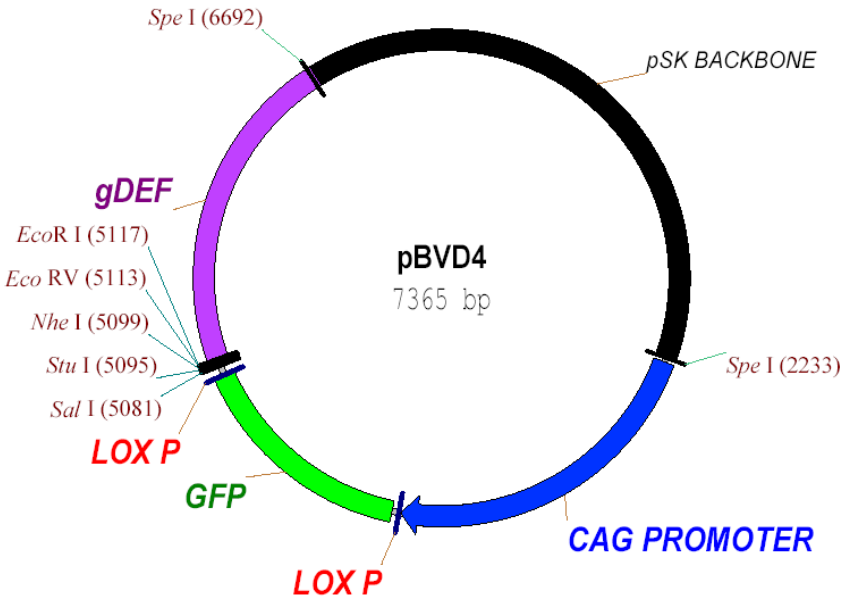

Kpn I (5106)
Sal I (5081)
LOX P
Afl II (5027)
Xba I (4799)
Not I (4789)
Pst I (4753)
Bsr GI (4647)
GFP
Age I (3925)
LOX P
CAG PROMOTER
pSK BACKBONE
pBVD3
5758 bp
Sac I (2207)
Not I (2220)
Xba I (2227)
Pst I (2255)

Figure 6.

A.


Sal I
Not I
Mouse β-globin Terminator

B.


Spe I (6692)
pSK BACKBONE
gDEF
EcoR I (5117)
Eco RV (5113)
Nhe I (5099)
Stu I (5095)
Sal I (5081)
LOX P
GFP
LOX P
pBVD4
7365 bp
Spe I (2233)
CAG PROMOTER