Hypoxia Inducible Double Plasmid System for Myocardial Ischemia Gene Therapy

Yi Tang, MaShira Jackson, Keping Qian, M. Ian Phillips

Abstract—Coronary artery disease frequently involves repeated bouts of myocardial ischemia. To automatically up-regulate the cardioprotective transgenes under hypoxic ischemia, a “vigilant vector” gene therapy system was developed and tested in a rat embryonic myocardial cell line (H9c2). In the vigilant vector, a hypoxia response element-incorporated promoter was used as a switch to turn on the gene expression in response to hypoxic signal. Furthermore, a novel double plasmid system was designed to elevate the potency of the vigilant vector. Instead of putting the promoter and the reporter gene in the same plasmid (single plasmid system), we separated them into two plasmids: the transactivator plasmid and reporter plasmid (double plasmid system). The hypoxia response element (HRE)-incorporated promoter increased the expression of a chimeric transcription factor consisting of the yeast GAL4 DNA binding domain and the human nuclear (transcription) factor-κB (NF-κB) p65 activation domain. The powerful chimeric regulator binds specifically to the upstream activating sequence for GAL4 in the reporter plasmid and activates the transcription of the transgene. Our experiments showed that the HRE-mediated expression could quickly increase 2.08 ± 0.75-fold within 6 hours of hypoxia and further augmented 7.12 ± 1.52-fold when the hypoxia condition was prolonged to 24 hours. The hypoxia-inducible double plasmid system dramatically amplified the transgene expression under both hypoxia and normoxia by 412.79 ± 185.27-fold and 205.35 ± 65.44-fold, respectively, relative to the single plasmid system. From these results, we concluded that this hypoxia inducible double plasmid system could be used therapeutically to switch on genes that have proven beneficial effects in myocardial ischemia. (Hypertension. 2002; 39[part 2]:695-698.)

Key Words: hypoxia ▪ myocardial ischemia ▪ transcription ▪ gene therapy

The majority of myocardial ischemic episodes in coronary artery disease are asymptomatic (review1). Because the ischemias are silent, patients miss timely and proper treatments. In order to provide cardioprotection against silent myocardial ischemia, chronic hypoperfusion, and prolonged postischemic dysfunction, we are developing a vigilant gene therapy system whose expression level can be automatically up regulated by hypoxic ischemia. Under hypoxia the transcription of dozens of genes is activated to maintain systemic and local oxygen homeostasis, including erythropoietin,2 vascular endothelial growth factor,3 glycolytic enzymes,4 and α1β-adrenergic receptor.5 An important mediator of these responses is the interaction of a transcriptional complex termed hypoxia inducible factor-16,7 with its cognate DNA recognition site, typical 5′-NCGTGN-3′ known as the hypoxia response element (HRE). Inspired by these naturally hypoxia regulatory gene expressions, an HRE-incorporated promoter has been used in our system.

To increase the potency of the hypoxia-inducible vector, a novel double plasmid system was developed. We incorporated the HRE-containing promoter and reporter gene into a transactivator plasmid and reporter plasmid. In the transactivator plasmid, the hypoxia responsive promoter regulates the expression of a chimeric transcription factor consisting of the yeast GAL4 DNA binding domain8 and the human p65 activation domain9 from NF-κB. Under hypoxia, more chimeric regulators will be produced and will activate the reporter plasmid containing six copies of a 17bp GAL4 upstream activating sequence (UAS).10 Using the double plasmid strategy in combination with hypoxia inducible promoter, we successfully increased the expression level of the reporter gene under both low and normal oxygen conditions. This system is potentially useful for driving a therapeutic transgene to provide protection in myocardial ischemia.

Methods

Construction of Plasmids

Single Plasmid System
Plasmid gene luciferase (pGL) with HRE and simian virus 40 promoter (pGL-HRE/SV40, kindly provided by Dr Semenza,4 the...
Figure 1. Diagram of plasmids. A, Single plasmid system. A 68bp human enolase 1 hypoxia response element (HRE) was inserted into upstream of the SV40 promoter in the pGL-SV40 plasmid to create pGL-HRE/SV40. B, Double plasmid system. The transactivator plasmid, pGS-SV40 and pGS-HRE/SV40 express a chimeric transcription factor consisting of the yeast GAL4 DNA binding domain (DBD) and the human p65 activation domain (AD) under the control of SV40 or HRE/SV40 promoter, respectively. The report plasmid, pGene-Luc, encodes luciferase driven by six copies of a 17bp GAL4 upstream activating sequence (UAS) and an adenosine E1b TATA box.

Johns Hopkins University, Baltimore, MD) was derived from pGL-SV40 (pGL2-Promoter, Promega) by insertion of a 68bp human enolase 1 hypoxia response element (HRE) sequence (416 to 439, Genebank: X16287) into 5′ flank of the simian virus 40 (SV40) promoter (Figure 1A).

**Double Plasmid System**

1. The transactivator plasmid: to generate plasmid gene switch (GS), pGS-SV40, and pGS-HRE/SV40, the SV40 and HRE/SV40 fragments were amplified by polymerase chain reaction (PCR) from pGL-SV40 and pGL-HRE/SV40, respectively, with the primer pairs designed with 5′ SbfI or 5′ SacI sites on the ends. The PCR products were digested by SbfI and SacI and ligated to SbfI/SacI-digested pGS-CMV plasmid to replace the CMV enhancer/promoter. pGS-CMV, a generous gift from Dr. Sean M Sullivan, University of California, Florida, Gainesville, FL, expresses a chimeric transcription factor consisting of the yeast GAL4 DNA binding domain (amino acids 1 to 93) and the human p65 activation domain (amino acids 283 to 349, Genebank: X16287) into 5′ flank of the simian virus 40 (SV40) promoter (Figure 1A).

**Results**

Time course of HRE directed hypoxia response in myoccardial cells. Two μg/well pGL-HRE/SV40 was transfected into H9c2 cells along with 100ng/well pRL-TK control plasmid in 60-mm dishes. Twenty-four hours after transfection, duplicate plates were incubated in either 20% or 1% O2 for 2, 4, 6, and 24 hours before preparation of cell lysates. Results are expressed as a ratio of firefly luciferase activity over Renilla luciferase activity (relative luciferase activity). The ratio of relative luciferase activity in cells at 1% O2 compared with 20% O2 was also calculated to determine induction by hypoxia (mean±SD; n=3 to 4 independent experiments; *P<0.05 1% O2 versus 20% O2. Two-way ANOVA was used to determine statistical significance).

**Cell Culture and Hypoxic Treatment**

A rat embryonic cardiac myoblast cell line, H9c2 (ATCC: CRL1446), was maintained in Dulbecco’s modified Eagle’s medium supplemented with sodium pyruvate and 10% fetal bovine serum. Cells were cultured under normoxic conditions (5% CO2, 20% O2, 75% N2) in a humidified incubator at 37°C. For hypoxic treatment, sealed chambers were incubated at 37°C under 20% O2. However, pGL-SV40 did not show hypoxic induction for any time periods (data not shown). HRE could slightly increase the activity of SV40 promoter even under normoxia by 1.74±0.10-fold, which was similar to the results from Dr Semenza.

**The Expression of SV40 Double Plasmid System**

We next examined whether the double plasmid system could amplify the activity of SV40 promoter. In the double plasmid system, the reporter expression elevated in a dose-dependent manner with increasing amounts of transfected transactivator plasmid (Figure 3). Without the transactivator plasmid, the expression level of the reporter plasmid was 33.6±3.8% of that of the single plasmid system (pGL-SV40). Small amounts of transactivator plasmid (10 ng) increased the expression of reporter plasmid by 3.18±0.03-fold relative to single plasmid system (pGL-SV40). The maximal expression
of the double plasmid system, achieved by adding 750 ng pGS-SV40, was 410.56 ± 84.42-fold over that of the single plasmid system (pGL-SV40). The reporter transcription appeared to plateau at higher levels of transfected transactivator plasmid, suggesting that the available GAL4 binding sites or some other component of the system may have become limiting.

**The Expression of HRE/SV40 Double plasmid System under Normoxia and Hypoxia**

The absolute expression levels of HRE/SV40 double plasmid system under both low and normal oxygen conditions were highly increased compared with that of single plasmid system (pGL-HRE/SV40) (Figure 4). Ten nanograms per well pGS-HRE/SV40 increased the reporter expression by 58.78 ± 21.65-fold under hypoxia, and 8.83 ± 2.23-fold under normal oxygen relative to the expression of single plasmid system (pGL-HRE/SV40) at 20% O2. The reporter expression can be further increased up to 412.79 ± 185.27-fold at 1% O2 and 205.35 ± 65.44-fold at 20% O2 by 100ng/well pGS-HRE/SV40 relative to the expression of the single plasmid system (pGL-HRE/SV40) at 20% O2.

**Discussion**

Several inducible gene expression systems have been developed. Most of them need exogenous compounds as inducers, such as tetracycline,12 ecdysone,13 rapamycin,14 and mifepristone.15 By utilizing the HRE-incorporated promoter, in this study we have designed a vigilant vector that could be selectively activated by an endogenous pathophysiological signal, hypoxia.

The HRE fragment used in our study has been proved to be sufficient to direct hypoxia-induced transcription in Hep3B cells.4 However, HREs have distinct hypoxic inducible ability in different cell lines.5,16 It has not been proven if the HRE can direct hypoxia response in myocardial cells. Here we demonstrated that the HRE could increase the gene expression up to 8-fold under hypoxia in cardiac myocytes. Moreover, our results showed that this hypoxia-induced expression could happen within 6h, which fit in with the therapeutic time window of myocardial ischemia.17

Since the potency of gene expression is pivotal for effective gene therapy, we further improved the vigilant vector with a novel double plasmid system. Instead of constructing the promoter and the reporter gene in the conventional way (single plasmid system), we separated the promoter into a transactivator plasmid and the reporter gene into a reporter plasmid (double plasmid system). The fusion protein, GAL4/p65, produced by the transactivator plasmid has strong transcriptional activity. We demonstrated that the double plasmid system dramatically amplified the function of SV40 promoter by up to 400-fold. This system could provide a promising way to improve the activity of other ubiquitous and tissue-specific promoters.

We also demonstrated that the HRE-incorporated double plasmid system could elevate the expression levels of reporter gene under both normal and low oxygen conditions up to 200- and 400-fold, respectively, and maintained 2- to 7-fold hypoxia induction ratio. This expression profile is different from other hypoxia-inducible constructs,18,19,20 which have low basal level under normoxia but substantial increase under hypoxia. Those vectors will be useful for tumor-specific gene therapy to minimize the undesired side effects in normal tissue and only aim to hypoxic tumor tissue. However, in coronary artery disease, there is a need for constant basal treatment and increased protection during ischemic attacks. The expression profile of the hypoxia responsive double
plasmid system is more suitable to myocardial ischemia disease. In addition, the double plasmid system also provides the advantage to achieve different expression levels and hypoxia induction folds by adjusting the amount of transactivator plasmid. This gives more flexibility to gene therapy when different subgroups of patients may require different doses of the therapeutic genes. This hypoxia sensitive vector could be used with genes that have proven beneficial effects in ischemia, such as superoxide dismutase, heme oxygenase, and antisenses to angiotensin II type 1 receptor, β-1 adrenergic receptor, and angiotensin-converting enzyme.

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References
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