

System for Simultaneous Tissue-Specific and Disease-Specific Regulation of Therapeutic Gene Expression

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ABSTRACT

Gene therapy has been proposed as an alternative strategy for treating nongenetic disorders, such as cancer and coronary artery disease. However, for many of these types of diseases, the therapeutic genes must be tightly regulated, as extensive toxicity and pathology can result if their expression is not adequately controlled. Toward this end, we have developed a regulatory system in which the expression of a therapeutic transgene is controlled simultaneously by both a tissue-specific promoter and a disease-specific promoter. Thus, the transgene of interest will be expressed in a given cell only if both of these promoters are active. Unlike many other transgene-regulatory systems that have been previously developed, this system does not require the persistent expression of any foreign genes that could provoke an immune response or lead to toxicity. As proof of concept, we synthesized a construct harboring the *lacZ* transgene that is under the control of both the hepatocyte-specific human α_1 -antitrypsin promoter and the zinc-inducible mouse metallothionein promoter. We show that reporter gene expression from this construct is regulated in both a hepatocyte-specific and zinc-regulated manner, as reporter gene expression occurs only in hepatocyte-derived cells that have been exposed to zinc. The improved regulation offered by our system would facilitate the targeting of transgene expression to sites of disease in the body and spare healthy tissue, thereby considerably enhancing the therapeutic window of gene therapy.

OVERVIEW SUMMARY

We have developed a system in which the expression of a therapeutic transgene is controlled simultaneously by both a tissue-specific promoter as well as a disease-specific promoter. The specificity of therapeutic gene expression provided by our system would not only improve the safety of the gene therapy modality but would also open many avenues for novel approaches in treating a wide variety of disorders refractory to currently available therapies.

INTRODUCTION

GENE THERAPY may some day prove to be a powerful modality in treating a wide range of nongenetic disorders. For this approach to attain its potential, it will be necessary to develop a means of precisely targeting the expression of therapeutic genes to diseased tissue. To fulfill this need, we have pursued the development of a system that would place a trans-

gene under the control of both a tissue-specific promoter and a disease-specific promoter, thereby facilitating simultaneous tissue-specific and disease-specific regulation of therapeutic gene expression.

The need for such stringent regulation can be illustrated by considering the proposed use of gene therapy in treating coronary artery disease. In this approach, genes encoding angiogenesis factors, such as vascular endothelial growth factor (VEGF), are transferred to the heart in order to stimulate the growth of new blood vessels to restore perfusion to the ischemic myocardium (reviewed in Isner, 2002). However, it would be desirable to restrict the expression of these therapeutic genes to the heart, as the expression of these potent genes in tissue outside of the heart could be deleterious. Indeed, excessive neovascularization in noncardiac tissue has been implicated in tumor formation and metastasis (reviewed in Kerbel, 2000), diabetic retinopathy (reviewed in Spranger and Pfeiffer, 2001), and rheumatoid arthritis (reviewed in Brenchley, 2001). Moreover, in addition to targeting the heart, it would also be desirable to restrict therapeutic gene expression to sites of ischemia within

the heart rather than allow nonspecific expression throughout the myocardium. In support of this notion, constitutive overexpression of VEGF in the nonischemic heart has been shown to lead to hemangioma formation, heart failure, and mortality within weeks of gene transfer in mice (Lee *et al.*, 2000). This pathologic outcome has also been observed in rats, as the delivery of an unregulated VEGF gene to the heart led to vascular anomalies (Schwarz *et al.*, 2000). A safe and viable strategy for treating coronary artery disease through gene therapy, then, will require targeting of the expression of an angiogenesis-promoting gene not only to the myocardium but also to the regions of the myocardium that are ischemic.

Coronary artery disease is only one example of why a system that would offer precisely targeted therapeutic gene expression is needed. Another clinical application in which such a system would be of value is the treatment of tumors through the delivery of cytotoxic genes (reviewed in McCormick, 2001). To target the expression of toxic genes to tumor cells, tumor-selective promoters have been used by investigators, but the vast majority of these promoters are not truly tumor specific and, in fact, many of these tumor-selective promoters are only tissue specific (reviewed in Nettelbeck *et al.*, 2000). In contrast, antitumor gene therapy in which a therapeutic gene is simultaneously regulated by both a tissue-specific promoter and a disease-specific promoter would be endowed with a considerably enhanced therapeutic window, as the therapy would be more precisely targeted to tumors and would spare normal tissue.

To illustrate, a transgene could be placed under the simultaneous control of both a tissue-specific promoter active in the tumor cells in question as well as a promoter that is active in the tumor cells but not active in normal cells of the same histological origin. For example, most melanoma cells express Fas ligand (FasL), whereas normal melanocytes do not express FasL under normal conditions (Hahne *et al.*, 1996). Hence, by using both a melanocyte-specific promoter and the FasL promoter to simultaneously regulate the expression of a toxic gene, melanoma cells could be exclusively targeted for destruction, while normal melanocytes and nonmelanocyte cells expressing FasL will be spared. This strategy can be generalized to other tumors, as there are many other promoters besides FasL that have been observed to be active in tumor cells and inactive in normal tissue of the same histological origin, such as telomerase (Koga *et al.*, 2001; Plumb *et al.*, 2001; Gu *et al.*, 2002), angiogenic growth factors (Koshikawa *et al.*, 2000), hypoxia-regulated factors (Dachs *et al.*, 1997; Shibata *et al.*, 2000), cyclooxygenase 2 (COX-2) (Casado *et al.*, 2001; Wesseling *et al.*, 2001; Yamamoto *et al.*, 2001), and many others.

A gene delivery approach in treating patients infected with the hepatitis C virus (HCV) represents yet another area in which more precisely regulated transgene expression could prove valuable. To illustrate, a vector harboring a cytotoxic gene could be delivered to patients in order to eliminate any HCV-infected cells. Expression of the cytotoxic gene could be selectively targeted to the infected cells by placing the gene under the control of a promoter activated by infection with HCV, such as the promoters for interleukin 8 (Polyak *et al.*, 2001), insulin-like growth factor II (Lee *et al.*, 2001), c-Myc (Ray *et al.*, 1995), or monocyte chemoattractant protein 1 (Meng Soo *et al.*, 2002). However, because these promoters can also be active in nonhepatocytes in the absence of HCV infection, it will be necessary to add the reg-

ulation offered by a hepatocyte-specific promoter in order to precisely target the cytotoxic gene expression to HCV-infected hepatocytes. Many hepatocyte-specific promoters are available for use toward this end, such as promoters for the albumin (Kramer *et al.*, 2003), α_1 -antitrypsin (Ciliberto *et al.*, 1985), and factor IX (Greenberg *et al.*, 1995) genes. The ability to regulate a therapeutic gene by both a hepatocyte-specific promoter and an HCV-activated promoter together would therefore engender the selective targeting of HCV-infected hepatocytes for destruction. This strategy could also be applied toward the treatment of hepatitis B virus (HBV) infections by selectively targeting cytotoxic gene expression to HBV-infected hepatocytes.

Simultaneous tissue-specific and disease-specific regulation of transgene expression, then, would provide a considerably enhanced therapeutic window for gene therapy approaches in treating a wide variety of nongenetic disorders. In this study, we present the development of a system that permits such a precise level of regulation and demonstrate proof of concept in a hepatoma cell culture model.

MATERIALS AND METHODS

Plasmids

The plasmid pCMVnlsLacZ was constructed by cloning the blunted *Sall-NcoI* fragment from pAAV-Pless-nlsLacZ (Nakai *et al.*, 2000), which carries the *lacZ* gene tagged with a sequence encoding a nuclear localization signal (NLS), into the blunted *XbaI* site of pHMCMV6 (Mizuguchi and Kay, 1999), which is also referred to as pCMV in this study. To generate pSS \times 2, pSS \times 3, and pSS \times 4, a *NotI-KpnI*-flanked oligonucleotide with the sequence 5'-AAGGTAAGTAGTCTGACAAGGTAAGT-3', 5'-AAGGTAAGTAGTCTGACAAGGTAAGTAGTCTGACAAGGTAAGT-3', or 5'-AAGGTAAGTAGTCTGACAAGGTAAGTAGTCTGACAAGGTAAGT-3', respectively, was ligated with the *NotI-KpnI* fragment of pCMVnlsLacZ. These sequences contain two, three, or four copies of a 5' splice site consensus sequence, separated from each other by 8 bp of stuffer sequence, as described previously (Barksdale and Baker, 1995).

To create pLox(SS \times 2)Lox, a *KpnI-AflIII*-flanked oligonucleotide with the *loxP* site sequence (Hoess *et al.*, 1982) was first ligated with the *KpnI-AflIII* fragment of pSS \times 2, which produced the plasmid p(SS \times 2)Lox, in which the *loxP* site is downstream of the 5' splice site pair. Because efficient Cre-mediated recombination requires that there be at least 82 bp in between two *loxP* sites (Hoess *et al.*, 1985), it was necessary to place a 250-bp stuffer fragment upstream of the pair of 5' splice sites to further increase the distance between the two *loxP* sites. This was achieved by first ligating a *Clal-Sall*-flanked oligonucleotide linker, bearing both a *NotI* site and a *BspEI* site, with the *Clal-Sall* fragment of pAAV-Pless-nlsLacZ. A *BspEI*-flanked oligonucleotide carrying the *loxP* site sequence was then cloned into the *BspEI* site of the resulting clone. Finally, a fragment carrying the *loxP* site with the 250-bp stuffer DNA was cut out of this resulting clone with *NotI* and then cloned into the *NotI* site of p(SS \times 2)Lox. The resulting final construct, pLox(SS \times 2)Lox, has the 5' splice site pair, along with the 250-bp stuffer fragment, flanked by *loxP* sites.

To generate pCMVCre, the *cre* gene was first amplified by polymerase chain reaction (PCR) from the pCMV-nlsCre plasmid (Lieber *et al.*, 1996) with the following primers: 5'-ACATATCTAGAATGTCCAATTTACTGACCGTACACC-3' (sense) and 5'-CATATAGCGGCCGCATCGCCATCTTCCAGCAGGC-3' (antisense). The resulting product, which is flanked by *XbaI* and *NotI* sites, was digested with *XbaI* and *NotI* and then ligated with the *XbaI-NotI* fragment of pHM-CMV6. To generate pMETnlsLacZ, the mouse metallothionein promoter was first amplified by PCR from pmMT-1-StuI-HdIII (generous gift of R.D. Palmiter, Seattle, WA) with the following primers: 5'-AATACAATTGGGCGGTCCCGCTGTGCACAC-3' (sense) and 5'-AATAGCTAGCGTGAAGCTGGAGCTACGGAG-3' (antisense). The resulting product, which is flanked by *MfeI* and *NheI* sites, was digested with *MfeI* and *NheI* and then ligated with the *MfeI-NheI* fragment of pCMVnlsLacZ.

To create pTwoP(Met/haAT)Ceu and pTwoP(Met/haAT)Mfe, it was first necessary to generate the intermediate plasmids pMetLoxSS \times 2 and phAAT/Lox/T7/Cre. The pMET(nlsLacZ)LoxSS \times 2 plasmid was produced in two sequential cloning steps in which a *NotI-KpnI*-flanked oligonucleotide with the sequence 5'-AAGGTAAGTAGTCTGACAAGGTAAAGT-3' was inserted into pMETnlsLacZ and then a *NotI*-flanked oligonucleotide containing the *loxP* site sequence was inserted.

To create phAAT/Lox/T7/Cre, it was necessary first to clone a prokaryotic transcriptional terminator sequence just upstream of the *cre* gene before cloning in any *loxP* sites. The prokaryotic terminator sequence served to terminate any leaky transcription from the plasmid that may occur in bacteria. If leaky transcription of Cre were to occur, then this could lead to Cre-mediated recombination events on a *loxP* site-containing plasmid during propagation of the plasmid in bacterial cultures. To avoid this potential problem, an oligonucleotide carrying the bacterial T7 bacteriophage transcriptional terminator sequence (Harvey *et al.*, 1999) 5'-CTAGAGGCTCACCTTCGGGTGGCCTTCTGCGA-3' was cloned into the *XbaI* site of pCMVCre, such that the *XbaI* site was recreated at the 5' end of the insert. Next, the resulting clone was digested with *ApaI* and *XbaI* and ligated with an *ApaI-XbaI*-flanked oligonucleotide carrying the *loxP* site sequence, which produced pCMV/Lox/T7/Cre. Finally, to produce phAAT/Lox/T7/Cre, the human α_1 -antitrypsin (haAT) promoter was obtained by digesting pAAV-cFIX-16 (generous gift from K. Chu, Philadelphia, PA) with *MscI* and *SalI* and was then blunted and ligated with the blunted *MfeI-NheI* fragment of pCMV/Lox/T7/Cre.

It should be noted that in cloning phAAT/Lox/T7/Cre, it was observed that the DNA yields obtained from constructs carrying both the *cre* gene and *loxP* sites together in the same plasmid were extremely low, which has been observed previously by other investigators (Kaczmarczyk and Green, 2001). However, in constructs in which the T7 terminator sequence had been added, the DNA yields from plasmid preparations were restored to the levels normally seen with other plasmids. This phenomenon suggests that the leaky transcription of Cre in bacteria was responsible for the reduced DNA yield in constructs that had both the *cre* gene and a *loxP* site together.

To produce pTwoP(Met/haAT)Ceu, the blunted *BglII* fragment of phAAT/Lox/T7/Cre was ligated with the blunted ICeuI

fragment of pMET(nlsLacZ)LoxSS \times 2. To produce pTwoP(Met/haAT)Mfe, the blunted *BglII* fragment of phAAT/Lox/T7/Cre was ligated with the blunted *MfeI* fragment of pMET(nlsLacZ)LoxSS \times 2.

Cell culture

HeLa (cervical carcinoma) cells were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin, and 2 mM L-glutamine. Huh7 (hepatocellular carcinoma) cells were maintained at 37°C in 5% CO₂ in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin, 2 mM L-glutamine, and 0.1 mM nonessential amino acids.

Transfections and β -galactosidase assays

Eighteen hours before transfection, 3×10^5 cells were seeded per well in six-well plates. Transfection was performed using the SuperFect reagent as described by the manufacturer (Qiagen, Valencia, CA). In experiments in which only one plasmid was transfected into cells, 2.0 μ g of the plasmid was used. In experiments involving cotransfection, 1.5 μ g of each of the two plasmids was used. For experiments in which cells were treated with zinc, cells were grown in culture medium containing 150 μ M zinc sulfate following transfection. Twenty-four or 40 hr later, cell lysates were harvested and β -galactosidase protein levels in the lysates were measured by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). The limit of sensitivity for this assay was 6 pg/well, which corresponds to a 40-pg/mg concentration of total cellular protein under the conditions of our assay, as 150 μ g of cellular protein was added per well. To normalize for cellular protein levels, total protein in the cell lysates was measured with a DC protein assay kit (Bio-Rad, Hercules, CA), as described by the manufacturer.

RESULTS

Two-promoter system

In the two-promoter system, the expression of a therapeutic transgene is subjected to the control of two independent promoters, rather than just one promoter. As a result, the therapeutic gene can be regulated in both a tissue-specific and disease-specific manner simultaneously (Fig. 1). The therapeutic transgene of interest is placed under the control of a disease-specific promoter so that this gene will be transcribed only in cells bearing the particular disease state that activates this promoter. An example of such a disease-specific promoter would be a hypoxia-specific promoter, which is activated when cells are exposed to ischemic conditions.

However, a set of 5' splice sites placed together in tandem is inserted into the 3' untranslated region (3' UTR) of the transgene, just upstream of the polyadenylation signal. Placement of 5' splice sites at such a location has been shown to potentially suppress the translation of reporter gene mRNA (Barksdale and Baker, 1995; Gunderson *et al.*, 1998). As a result, even if the disease-specific promoter driving transcription of the transgene were to be active in a cell, the transgene will not be expressed

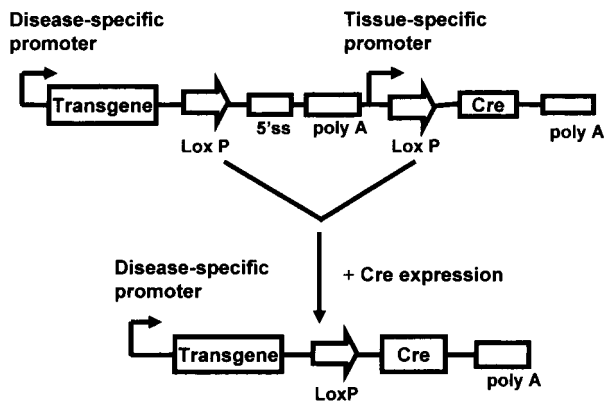


FIG. 1. Schematic representation of the two-promoter system. The tissue-specific promoter directs transcription of the gene encoding the site-specific recombinase Cre, while the disease-specific promoter directs transcription of the therapeutic gene. A set of 5' splice sites, designated here as "5'ss," are inserted into the 3' UTR of the therapeutic gene, which acts to block posttranscriptional processing of the therapeutic gene mRNA and thereby block translation of the therapeutic gene. "Lox P" designates the *loxP* sequence recognized by Cre. The *loxP* sites are inserted into the vector such that they flank both the 5' splice sites and the tissue-specific promoter.

because the mRNA transcript for this gene will not be translated.

The 5' splice sites placed in the 3' UTR are flanked by a pair of *loxP* sites oriented in parallel that are recognized by the site-specific recombinase Cre (Sternberg and Hamilton, 1981; Sternberg *et al.*, 1981), and the gene encoding the Cre protein is placed under the control of a tissue-specific promoter. Consequently, in cells of the tissue type in which the tissue-specific promoter is active, Cre will be expressed and will then mediate site-specific recombination about the *loxP* sites, leading to excision of the DNA encoding the inhibitory 5' splice site sequences. The therapeutic transgene will now be expressed if it is transcribed, as the inhibitory 5' splice sites will no longer be present. Thus, in this system, both the disease-specific promoter and tissue-specific promoter must be active in a cell in order for the transgene to be expressed, because the disease-specific promoter must be active so that the transgene can be transcribed and because the tissue-specific promoter must be active so that Cre can be expressed and remove the DNA encoding the inhibitory 5' splice sites that block translation of the transgene.

As an additional feature of the system, the promoter for the *cre* gene shall also be located between the *loxP* sites so that *cre* expression will result in its own ablation, as the expressed Cre protein will excise the promoter away from the *cre* gene. As a result, *cre* expression will only be transient, lasting long enough to excise the inhibitory 5' splice sites but not long enough to lead to any of the potentially undesirable effects of prolonged *cre* expression. This self-inactivation feature is of value because the Cre protein is foreign to the human body, and long-term *cre* expression could provoke a cellular immune response against *cre*-expressing cells. In addition, persistent *cre* expression in a cell may lead to pathologic effects and, indeed, prolonged *cre* expression has been reported to lead to recombination events

within the cellular genome itself (Thyagarajan *et al.*, 2000) as well as DNA damage, chromosomal abnormalities, and aberrant cell proliferation (Loonstra *et al.*, 2001).

Determination of optimal number of 5' splice sites needed for maximal suppression of transgene expression

In building the two-promoter system, it was necessary first to determine the optimal number of 5' splice sites that would maximally suppress gene expression on placement in the 3' UTR. A pair of 5' splice sites inserted into the 3' UTR of a reporter gene has been previously observed to significantly inhibit expression (Barksdale and Baker, 1995; Gunderson *et al.*, 1998), but it is unknown whether more 5' splice sites can even further reduce the level of reporter gene expression. To explore this issue, constructs harboring a reporter gene with two, three, or four 5' splice sites placed in the 3' UTR were generated (Fig. 2A) and transiently transfected into HeLa cells. All these constructs were observed to yield expression levels below the detection level of the assay (Fig. 2B).

Because the inhibitory effect of 5' splice sites on posttranscriptional processing has been reported only in HeLa cells so far (Barksdale and Baker, 1995; Gunderson *et al.*, 1998), we investigated whether the inhibition occurs in another cell line. Constructs carrying different numbers of 5' splice sites were transfected into Huh7 cells and, in contrast to the results obtained in HeLa cells, complete suppression of reporter gene expression was observed only when two 5' splice sites are placed in the 3' UTR (Fig. 2C). A pair of 5' splice sites, then, seems to be optimal in suppressing gene expression in both HeLa and Huh7 cells.

It is unclear why there exists a disparity in the degree of suppression conferred by three or four 5' splice sites in HeLa cells as compared with Huh7 cells. One possible explanation is that perhaps all that is needed for efficient suppression of reporter gene expression is a pair of 5' splice sites, and so the assembly of splicing factors onto any excess 5' splice sites does not augment the suppressive effect but rather depletes the supply of available splicing factors. In cells in which the supply of available splicing factors is limited, such as perhaps in Huh7 cells, there may not be adequate amounts of splicing factors to bind all the 5' splice sites, thereby precluding efficient suppression of reporter gene expression in those cells. Another possible explanation is that the splicing process or other steps in posttranscriptional processing may be regulated differently in Huh7 cells as compared with HeLa cells.

Cre-mediated rescue of transgene expression

To test whether Cre-mediated site-specific recombination could be used to rescue transgene expression from the effects of inhibitory 5' splice sites, we synthesized a construct in which a *loxP*-flanked pair of 5' splice sites was inserted into the 3' UTR of the reporter gene (Fig. 3A). This construct, pLox (SS×2)Lox, was then cotransfected into Huh7 cells with a Cre-expressing plasmid, pCMVCre. A high level of reporter gene expression was observed, which was approximately 15% of that observed on transfection with pCMVnlsLacZ, a reporter gene construct devoid of any 5' splice sites in the 3' UTR. This result confirms that Cre expression could indeed rescue transgene

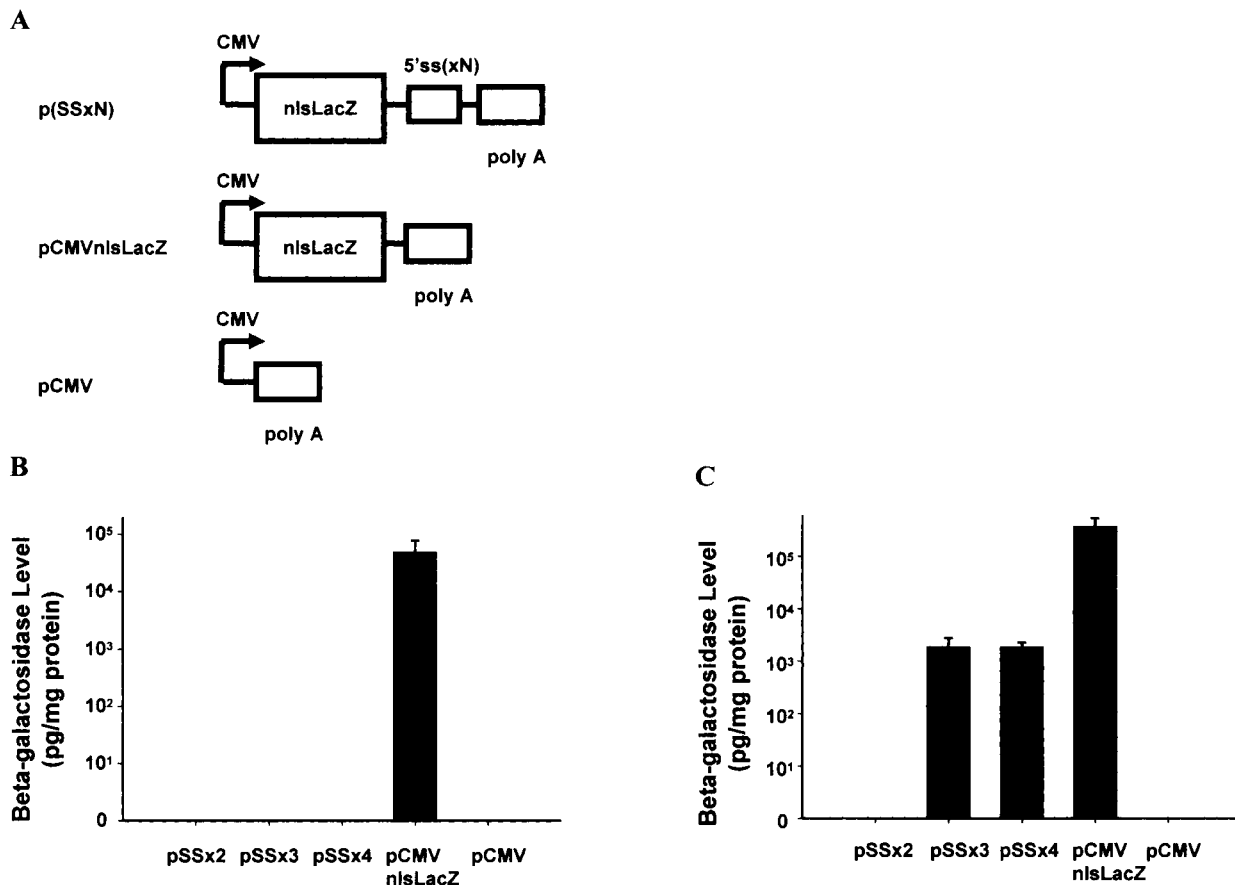


FIG. 2. Optimal suppression of reporter gene expression by 5' splice sites in the 3' UTR. **(A)** Schematic representation of the reporter constructs. Two, three, or four 5' splice site consensus sequences, designated here as "SS(XN)," are inserted into a reporter gene's 3' UTR in pSS×2, pSS×3, and pSS×4, respectively. The plasmid pCMVnlsLacZ does not have any 5' splice sites in its 3' UTR and serves as a positive control, whereas pCMV lacks a reporter gene and serves as a negative control. **(B)** Optimal suppression by 5' splice sites in HeLa cells. Constructs were transfected into HeLa cells, and nlsLacZ expression was assayed by a β -galactosidase ELISA 24 hr later. **(C)** Optimal suppression by 5' splice sites in Huh7 cells. Constructs were transfected into Huh7 cells, and nlsLacZ expression was assayed by a β -galactosidase ELISA 24 hr later. The data shown are averages of triplicates \pm standard deviation.

expression from the posttranscriptional blockade mediated by the 5' splice sites (Fig. 3B). Similar results were obtained in HeLa cells (data not shown).

There are several possible reasons why cotransfection with the Cre plasmid with the *loxP* reporter construct does not fully restore reporter gene expression levels to those observed on transfection with a reporter gene construct that is already devoid of any 5' splice sites. First, because only a minority of cells are successfully transfected with the SuperFect reagent, it is possible that only a small subset of the cells transfected with the *loxP* reporter construct are successfully cotransfected with the Cre plasmid. Second, the 24 hr between transfection and performance of the reporter assay may not have been enough time for Cre to be fully expressed and carry out the excision of the *loxP*-flanked 5' splice sites. Third, the distance between the two *loxP* sites in this construct was approximately 300 bp and may have been smaller than is optimal for Cre-mediated site-specific recombination, as a relationship between distance and efficiency of Cre activity has been previously reported (Hoess

et al., 1985). In any case, the data here show that the Cre-*loxP* system can be used successfully to excise the DNA encoding the inhibitory 5' splice sites and rescue transgene expression.

As a negative control, pLox(SS×2)Lox was cotransfected into Huh7 cells with only the negative control plasmid pCMV. The level of reporter gene expression obtained was below the detection limit of the assay. This result indicates that the mere flanking of the inhibitory 5' splice sites with *loxP* sites does not undermine the inhibitory effect of the splice sites or lead to spontaneous recombination and excision in the absence of the Cre protein (Fig. 3B).

Simultaneous tissue-specific and condition-specific regulation of transgene expression

As proof of concept for the two-promoter system, we synthesized a construct in which a reporter gene was controlled by both the hepatocyte-specific human α_1 -antitrypsin (hAAT) promoter (Ciliberto *et al.*, 1985) and the zinc-inducible mouse met-

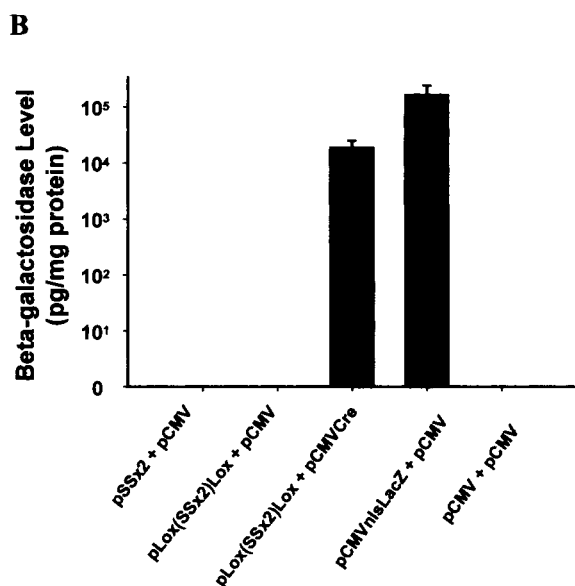
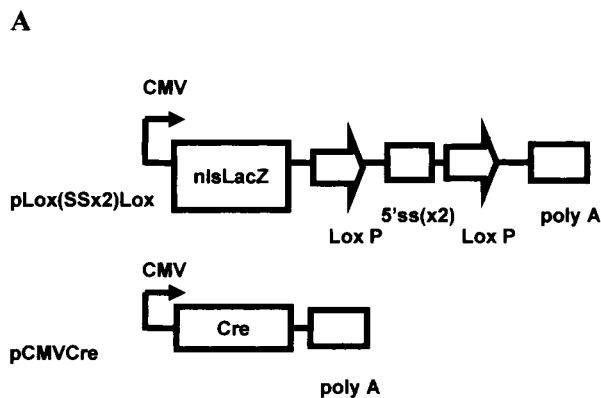


FIG. 3. Cre-mediated rescue of reporter gene expression. (A) Schematic representation of pLox(SS \times 2)Lox and pCMVCre. In the plasmid pLox(SS \times 2)Lox, two 5' splice sites are inserted into the 3' UTR but are flanked by loxP sites. (B) Restoration of nlsLacZ expression by coexpression of Cre. Constructs were transfected into Huh7 cells, and nlsLacZ expression was assayed by a β -galactosidase ELISA 24 hr later. The data shown are averages of triplicates \pm standard deviation.

allothionein promoter (Stuart *et al.*, 1984) simultaneously (Fig. 4A). The metallothionein promoter can serve as a surrogate for a disease-specific promoter for the purpose of testing the validity of the two-promoter system, as this promoter can be activated by subjecting cells to a specific condition, which in this case is incubation in zinc. In this construct, p(Met/hAAT)(nlsLacZ)Ceu, the *cre* gene was placed under the control of the metallothionein promoter (Fig. 4A). This construct was transfected into either Huh7 cells (hepatoma cell line) or HeLa cells (cervical carcinoma cell line) that had been cultured in the presence or absence of zinc sulfate.

Significant reporter gene expression was observed only in transfected Huh7 cells treated with zinc (Fig. 4B). In Huh7 cells cultured without zinc or in HeLa cells cultured with zinc, trans-

fection with the construct led to only minimal expression levels that were not different from those obtained by transfection with the negative control plasmid pCMV (Fig. 4B). Thus, for the reporter gene to be expressed in a given cell, both the hAAT promoter and the metallothionein promoter had to be active, as cells in which only one of the two promoters was active did not express the transgene.

This result contrasted with that obtained by transfection with pCMVnlsLacZ, a construct in which the reporter gene is driven by the constitutively active cytomegalovirus (CMV) promoter. Transfection with this construct led to high expression levels regardless of cell type or of the presence or absence of zinc in the culture medium (Fig. 4B).

Alternative two-promoter system

One possible strategy for delivering a therapeutic gene regulated by the two-promoter system would be through the ad-

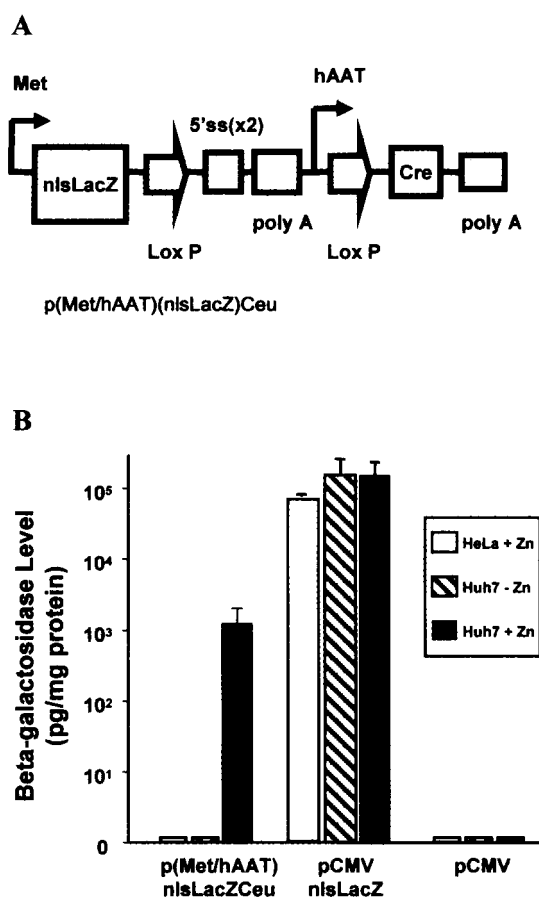


FIG. 4. Regulation of reporter gene expression by the two-promoter system. (A) Schematic representation of p(Met/hAAT)(nlsLacZ)Ceu. The mouse metallothionein promoter, designated here as "Met," drives transcription of the nlsLacZ reporter gene, while the hAAT promoter, designated as "hAAT," drives transcription of the *cre* gene. (B) Simultaneous hepatocyte-specific and zinc-regulated reporter gene expression. The constructs were transfected into either Huh7 or HeLa cells, which were then cultured in media supplemented with or without 150 μ M zinc sulfate. Forty hours after transfection, nlsLacZ expression was assayed by a β -galactosidase ELISA. The data shown are averages of triplicates \pm standard deviation.

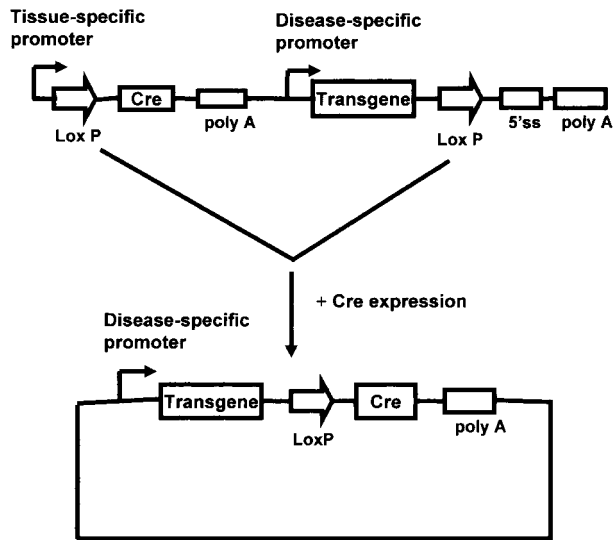


FIG. 5. Schematic representation of an alternative configuration of the two-promoter system. As in the original configuration, the tissue-specific promoter directs transcription of the gene encoding the site-specific recombinase Cre, while the disease-specific promoter directs transcription of the therapeutic gene. A set of 5' splice sites, designated here as "5'ss," are inserted into the 3' UTR of the therapeutic gene, which acts to block posttranscriptional processing of the therapeutic gene mRNA and thereby block translation of the therapeutic gene. In contrast to the original configuration of the two-promoter system, the transgene and its promoter, rather than the inhibitory 5' splice sites, are flanked by *loxP* sites and will be excised out of the plasmid on Cre expression.

ministration of naked plasmid DNA, as has been performed in gene therapy for the heart (Losordo *et al.*, 1998), skeletal muscle (Acsadi *et al.*, 1991), lung (Gill *et al.*, 2001), brain (Schwartz *et al.*, 1996), and skin (Choate and Khavari, 1997). However, evidence suggests that bacterial sequences in the backbone of plasmids may lead to the silencing of transgene expression over time (Chen *et al.*, 2001, 2003; Ehrhardt *et al.*, 2003). In light of this concern, we devised an alternative configuration of the two-promoter system in which the transgene and its promoter, rather than the inhibitory 5' splice sites, are excised from the plasmid by Cre (Fig. 5). In this way, the therapeutic gene is not only liberated from the presence of the inhibitory 5' splice sites in its 3' UTR but is also physically separated from the rest of the plasmid harboring the bacterial backbone sequences that may lead to transgene silencing. It should be noted, however, that the mechanism of bacterial backbone-induced silencing is unclear and that if the effects are mediated *in trans* rather than *in cis*, then this alternative two-promoter system configuration will not elude the silencing effects of the bacterial backbone.

Testing of alternative two-promoter system

To test whether the two-promoter system with the alternative configuration could still confer simultaneous tissue-specific and condition-specific regulation of transgene expression, the construct p(Met/hAAT)(nlsLacZ)Mfe was generated (Fig. 6A). On transfection of this construct into either Huh7 or HeLa cells

cultured with or without zinc, we observed that significant reporter gene expression was attained only in Huh7 cells cultured with zinc, indicating that the reporter gene required activation of both the hepatocyte-specific promoter and the zinc-activated promoter for expression to occur (Fig. 6B).

DISCUSSION

In this study, we show that a transgene can be placed under the control of two independent promoters through the two-promoter system, which would enable simultaneous tissue-specific and disease-specific regulation of a therapeutic gene of interest.

Although other approaches have been reported that superimpose an additional level of specificity on top of tissue-spe-

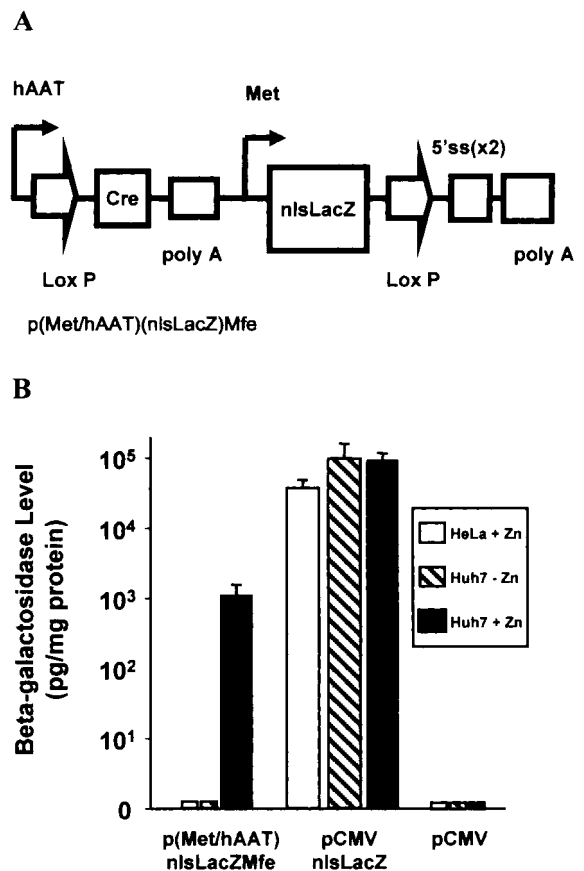


FIG. 6. Regulation of reporter gene expression by the alternatively configured two-promoter system. (A) Schematic representation of p(Met/hAAT)(nlsLacZ)Mfe. The mouse metallothionein promoter, designated here as "Met," drives transcription of the nlsLacZ reporter gene, while the hAAT promoter, designated as "hAAT," drives transcription of the *cre* gene. (B) Simultaneous hepatocyte-specific and zinc-regulated reporter gene expression. The constructs were transfected into either Huh7 or HeLa cells, which were then cultured in media supplemented with or without 150 μ M zinc sulfate. Forty hours after transfection, nlsLacZ expression was assayed by a β -galactosidase ELISA. The data shown are averages of triplicates \pm standard deviation.

cific regulation of transgene expression, these approaches are not without limitations or drawbacks. In one approach, artificial transcription factors, expressed from tissue-specific and/or cell cycle-regulated promoters, are used to modulate transcription from a promoter driving transcription of the transgene of interest. This strategy has been reported to facilitate simultaneous tissue-specific and cell cycle-regulated transgene expression (Jerome and Muller, 1998; Nettelbeck *et al.*, 1999). However, the major drawback to this strategy is the need to use foreign protein domains, as the DNA-binding domains of these artificial transcription factors cannot be endogenous, or else they will bind endogenous DNA sequences in the host genome. As a result, this approach risks provoking a cellular immune response against cells expressing these artificial transcription factors. In addition, there may be toxic effects from introducing artificial transcriptional regulatory proteins into cells, as has been previously observed (Furth *et al.*, 1994; Barton *et al.*, 2002).

In another approach, the promoter elements from one promoter are combined with that of another promoter. For example, hypoxia-regulated sequence elements have been inserted into a tissue-specific promoter to permit hypoxia-regulated and tissue-specific expression of transgenes (Prentice *et al.*, 1997; Modlich *et al.*, 2000; Hernandez-Alcoceba *et al.*, 2001; Ido *et al.*, 2001; Phillips *et al.*, 2002). However, the limitation of such an approach is the potentially narrow range of promoters for which this strategy can be applied, as combining promoter elements can lead to mutual interference with each other or increased leakiness of basal expression levels from the promoter. Moreover, such a strategy will have to be customized and explored for each individual promoter. In contrast, the two-promoter system is considerably more generalizable, as the two promoters are configured to act independently of each other.

Another approach involves the use of a site-specific recombinase fused to the ligand-binding domain of a steroid hormone receptor (Kellendonk *et al.*, 1999; Sohal *et al.*, 2001). This chimeric recombinase is placed under the control of a tissue-specific promoter to restrict its expression to a particular cell type, and the activity of the recombinase is controlled by the administration of an exogenous steroid hormone. On activation, this recombinase excises a *loxP*-flanked set of stop codons inserted upstream of the transgene of interest, thereby activating transgene expression. This approach, however, does not allow for disease-specific regulation, as the chimeric recombinase is activated by an exogenously administered drug rather than any disease state. Similarly, the lack of disease-specific regulation also limits the applicability of a system in which transgene expression is controlled in both a tissue-specific and tetracycline-regulated manner (Ghersa *et al.*, 1998).

It should be noted that the two-promoter system, too, may encounter potential limitations that need to be considered. For example, the inhibitory effects of placing 5' splice sites into the 3' UTR may not be universal in all tissues and may limit the organs in which the system will be functional. Moreover, because many promoters have some level of basal leakiness, it is possible that certain tissue-specific promoters will not be suitable for use in driving *cre* expression. This is the case because leaky expression of *cre* that exceeds a certain threshold may be sufficient to lead to the excision of the inhibitory 5' splice sites and may therefore undermine the tissue specificity of the sys-

tem. The only way to adequately address these concerns will be to investigate the regulatory capacity of the two-promoter system *in vivo* and to test many different promoter pairs in many different organs. The answers to these questions await the completion of studies already in progress.

Nevertheless, we have shown *in vitro* the development of a system that would permit the targeting of therapeutic gene expression in both a tissue-specific and disease-specific manner. Such stringent regulation of therapeutic genes would not only reduce the risk of pathologic effects resulting from nonspecific transgene expression but would also facilitate the use of transgenes with strong biological effects, as expression of the transgene could be precisely targeted to sites of disease in the body. Moreover, the improved therapeutic window would permit the use of higher doses of gene delivery vectors as well as the infusion of entire organs with a delivery vector rather than relying on direct injection into the pathologic sites within a given organ.

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