

Trapping of Mammalian Promoters by Cre-*lox* Site-Specific Recombination

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Abstract

One of the challenges in human genome research is to identify the promoter sequences which play a key role in the regulation of gene expression. We report here a new promoter trapping system for use with mammalian cells comprised of the following three steps: 1) Cloning of DNA fragments into a promoter-trapping vector, 2) integration of the trapping vector into a designated target in the mammalian genome using the Cre site-specific recombinase, and 3) screening of integrants for trapped promoter sequences by activation of the luciferase gene. To assess the efficiency of this system, *lox* trapping vectors containing sense *tk* promoter, antisense *tk* promoter, or a non-promoter sequence of the *neo* gene were employed. The resulting levels of luciferase activity of the site-specific integrants were measured directly. Luciferase activity of the integrants can be assayed under conventional culture conditions by simply replacing the culture medium with potassium phosphate buffer containing luciferin. Only those G418^r colonies carrying the *tk* promoter in the normal orientation exhibited a 21- to 35-fold increase in luciferase activity over that of the other integrants. These results indicate that this system is an effective means of trapping promoter sequences from random mammalian genomic DNA fragments.

Key words: promoter trapping; Cre-*lox* site-specific recombination; luciferase gene

1. Introduction

The identification and characterization of the disparate promoter elements found in complex genomes is an essential component of our understanding of such systems. Clearly, for such analysis, the isolation of the promoter sequences is an essential process. In general, promoters are identified by sequencing the genomic DNA which resides upstream of the transcription start site and isolated sequences are assessed by functional analysis. Methods of trapping functional promoter sequences directly from the genome have been previously published. In *Escherichia coli*, the promoter-trapping vectors are constructed with a promoterless antibiotic-resistance gene as a reporter. Random DNA fragments generated by digestion with an appropriate enzyme are ligated into a cloning site upstream of the antibiotic-resistance gene. The resulting plasmids are transformed into *E. coli*, and the trapped promoter sequences are evaluated on the basis of sensitivity to the antibiotic.¹ Although extrachromosomal

plasmids are not available in mammalian cells for promoter trapping, a number of trapping vectors have been constructed using a promoterless β -galactosidase (*β -gal*) gene as a reporter.²⁻⁷ The *β -gal* gene with a selectable marker (neomycin phosphotransferase (*neo*) gene) is usually transfected into embryonic stem (ES) cells using electroporation or retrovirus vectors. Only when the *β -gal* gene is downstream of the promoter sequences, G418 resistant ES cells are stained with X-gal. The ES cells selected in this manner are then injected into mouse embryos, where they are capable of developing into all the different cell types. Thus, the tissue- or stage-specific activity of each promoter can be easily examined. In addition, since this promoter-trapping system is able to destroy the gene which lies in downstream of the promoter, it is useful to obtain various types of mutant mice. However, this method is not adequate for trapping promoter sequences that are contained at interesting chromosomal loci.

In this report, we introduce a new system, in which mammalian genomic promoter sequences may be trapped irrespective of their chromosomal origin. X-gal staining is harmful to mammalian cells and thus it is difficult to use cells after X-gal staining for further studies. Consequently, the firefly luciferase gene has been used as a re-

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porter instead of the β -gal gene, allowing further cell culture after the enzyme assay *in situ* and reducing the time needed for analysis. There are several cloning sites for insertion of random DNA fragments in the region mapping 5' to the luciferase gene. In addition, the SV40 poly(A) signal has been placed upstream of the cloning sites in the trapping vector to repress illegitimate transcription from upstream DNA. The trapping constructs are unstable as episomal components in cells and are therefore integrated into the chromosomal *lox* site using the Cre-*lox* site-specific recombination of bacteriophage P1. This largely eliminates both the variability of gene expression due to random integration and the number of copies integrated into the genome resulting from conventional DNA transfection technologies.

Cre is a 38-kDa recombinase derived from bacteriophage P1 which mediates the site-specific recombination between 34-bp *lox* sites.⁸ Cre protein has been shown to perform efficient recombination reaction at *lox* sites not only in bacteria but also in eukaryotic cells.⁹⁻¹² Previously, a new *lox-neo* fusion gene was designed to facilitate the selection of integrative events in a wide variety of mammalian cells.^{13,14} In cell lines containing this target, Cre-mediated recombination of a promoter-ATG-*lox* targeting vector reconstructs a functional ATG-*lox-neo* fusion gene, and thus integration events can be selected directly by the sensitivity to the drug G418. Using CHO cell lines containing this *lox* target, almost all of the transformants (54 of 56 independent G418^r colonies) were simple single-copy integrants. Also, independent G418^r colonies obtained by site-specific recombination which are carrying a reporter gene exhibited nearly identical levels of gene expression.

We constructed a promoter-trapping vector as outlined above and isolated integrants resulting from the Cre-*lox* site-specific recombination. A thymidine kinase (*tk*) promoter and a promoterless DNA fragment were cloned into the trapping vector, and site-specific integrants were isolated. Only integrants containing the *tk* promoter in the normal orientation exhibited much more luciferase activity with the cell extracts as well as the intact cell colony growing on culture dishes. The latter case of quantitative luciferase assay was conducted using an Argus-50/CL. The integrant with a promoter-trapping vector exhibits background luciferase activity itself.

2. Materials and Methods

2.1. Plasmids and DNA constructions

All plasmids were constructed and prepared by conventional methods.¹⁵ The defective *lox-neo* gene fusion plasmid pSF1,¹³ the Cre expression vector pBS185¹² and the *lox* translational fusion targeting vector pBS226¹³

have been described previously. These plasmids were a generous gift of Dr. Brian Sauer (National Institutes of Health). Plasmid pSF1 was digested with *Bam*HI, DNA ends were trimmed with Klenow fragment of DNA Pol I (Pollk), ligated with a 2.0-kb *Pvu*II fragment of pTK4¹⁶ (obtained from Dr. Yasufumi Kaneda, Osaka University) containing the herpes simplex virus type I (HSV1) thymidine kinase (*tk*) gene yielding pSF73 (Fig. 1A). The promoter-trapping vector pSF86 containing a *lox* site (Fig. 1B) was constructed as follows. First, the 2.2 kb fragment (*Hind*III-*Not* I-*Sal* I-*Bam*HI-*Xho* I-*Not* I-*Sph* I polylinker-luciferase gene-*Pst* I-SV40 poly(A)-*Sph* I) was cloned into the *Sph* I and *Hind*III restriction sites of pBS226 to give pSF85. Second, pSF85 was digested with *Nde* I and *Hind*III, and a 300 bp fragment containing a SV40 poly(A) was inserted to generate pSF86. To assess the promoter-trapping capability of the system, a 648-bp *Sau*3AI fragment containing a *tk* promoter derived from plasmid pTK4 was cloned into the *Bam*HI site of pSF86. A 610-bp *Sau*3AI fragment derived from the *neo* gene was also cloned to serve as a promoterless control.

2.2. Cell lines and gene transfer

The defective *lox-neo* fusion gene in pSF73 was electroporated into the *Ltk*-mouse cells (obtained from RIKEN Cell Bank).¹⁷ Approximately 1 μ g of pSF73 DNA was electroporated into 1×10^7 cells in 0.8 ml HeBS buffer (20 mM Hepes pH7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose) using a single pulse of 1.8 kV at 25 μ F from a Gene Pulser (Bio-Rad Laboratories, Richmond, CA). After 2 days of gene transfer, stable transformants were selected in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and HAT (hypoxanthine, aminopterin, and thymidine). Colonies were counted 14 days after gene transfer and transformants for DNA analysis were cloned by limiting dilution in HAT medium.

DNA transfection into one of the single-copy integrants carrying the *lox-neo* target site, 73-16 with LIPOFECTAMINETM (GIBCO BRL, Gaithersburg, MD), a 3:1 (w/w) liposome formulation of 2,3-dioleoyloxy-*N*-[2(sperminocarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate and dioleoyl phosphatidylethanolamine was performed as outlined by the manufacturer, with the following modifications. 73-16 cells were seeded onto a 6-well culture dish and allowed to reach ~70% confluence. DNA was mixed with OPTI-MEM (GIBCO BRL), giving a final volume of 100 μ l. Ten microliters of LIPOFECTAMINE was added to 90 μ l of OPTI-MEM. The two aliquots were combined and incubated for 15 min at room temperature. The volume was brought to 1 ml by the addition of 0.8 ml of OPTI-MEM and then the mixture added to a single well of cells

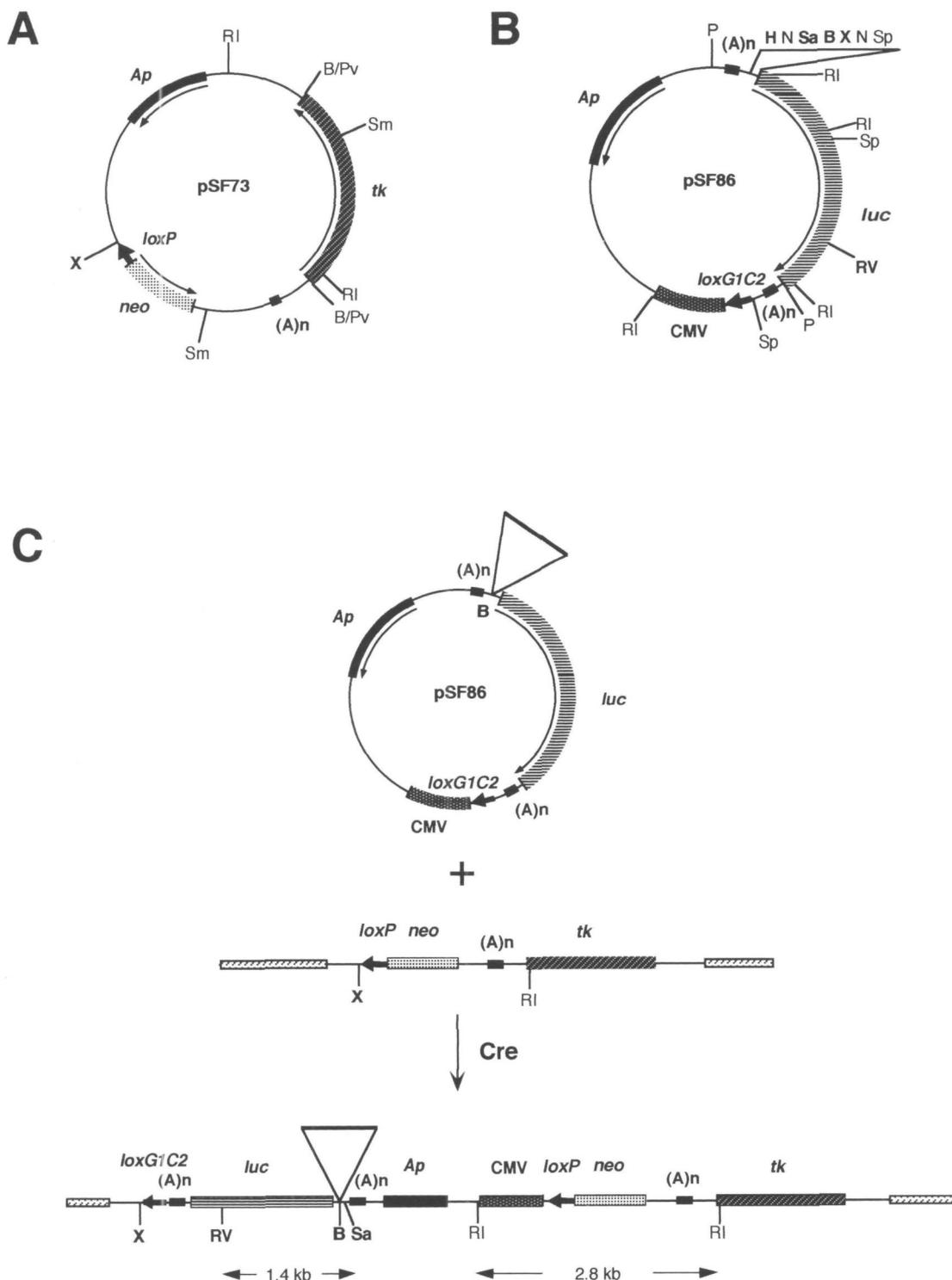


Figure 1. Site-specific promoter-trapping vectors and a scheme of reconstruction of a functional *lox-neo* gene by site-specific recombination. **A.** The *lox-neo* target vector pSF73 which contains *tk* gene as a selectable marker is shown. **B.** The promoter-trapping vector pSF86 is shown. **C.** The trapping vector pSF86 and the chromosomal target, the defective *lox-neo* construct of pSF73, are shown. Cre-mediated site-specific integration produces the structure shown and reconstructs a functional ATG-*lox-neo* fusion gene. The sizes of restriction fragments hybridizing with a CMV probe or with a luciferase gene probe are drawn in the figure. Restriction sites in bold type are unique: B, *Bam*HI; H, *Hind*III; N, *Not* I; P, *Pst* I; Pv, *Pvu* II; RI, *Eco*RI; RV, *Eco*RV; Sa, *Sal* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xho* I.

which were prewashed once with 2 ml of OPTI-MEM before the addition. Cells were incubated for 6 hr at 37°C and then had 1 ml of DMEM containing 20% fetal bovine serum added. The medium was replaced with 2 ml of fresh DMEM containing 10% fetal bovine serum at 18 hr post transfection. Two days after gene transfer, cells were trypsinized, replated onto four 10-cm culture dishes, and selected for growth in HAT medium containing 400 μ g/ml G418. Colony formation was scored 12 days afterwards and individual clones were cultured for further analysis.

2.3. DNA isolation and Southern hybridization

Genomic DNA of the integrants was prepared as described.¹⁸ To confirm that each G418^r colony represented a site-specific integration event and to determine the number of copies integrated, the transformants obtained by Cre-*lox* site-specific recombination were analyzed by Southern hybridization.¹⁹ Approximately 5 μ g of genomic DNA was digested with *Eco*RI and *Xho* I, electrophoresed on a 0.7% agarose gel and then blotted to GeneScreen (DuPont Co.). The blots were probed with a 700-bp hCMV to detect 3' site-specific recombination sites and to measure the copy number of the integrated trapping vector(s). In addition, digestion of genomic DNA with *Eco*RV and *Sal* I followed by hybridization probed with a 1.4-kb luciferase gene made it possible to structurally analyze the region of the *Bam*HI cloning site.

2.4. Luciferase assay

In vitro assay:

Cells ($\sim 1 \times 10^6$) were split into a 6-well culture dish and cultured for 3 days. Confluent cells were washed twice with phosphate-buffered saline (PBS(-)) and harvested in 1 ml of PBS(-) by scraping. The cells were centrifuged and resuspended in 100 μ l of extraction buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1.0 mM MgSO₄, 50 mM 2-mercaptoethanol, 2.5 mM ethylenediaminetetraacetic acid, and 0.125% NP-40, pH 7.2) by pipetting six times. Cell debris was removed by centrifugation in a microcentrifuge for 10 min at 4°C. A 10- μ l sample of extract was added to 70 μ l of 100 mM KPO₄ (pH 7.8) containing 5 mM ATP and 15 mM MgSO₄ in a 96-well white luminostrip (Labsystems). The luminostrip was placed in a single-photon counting and image processing system (Argus-50/CL, Hamamatsu Photonics K. K., Hamamatsu, Japan), and the reaction was initiated by the addition of 10 μ l of 10 mM luciferin (Sigma Chemicals, St. Louis, MO). Luciferase activity was quantitated by photon counting in complete darkness over a 5-min period. Total protein in the extracts was determined by coomassie brilliant blue G250 binding²⁰ with protein assay reagent (Bio-Rad Laboratories).

In vivo assay:

About 50 cells were plated onto a 10-cm culture dish to measure the luciferase activity directly. Following 2 weeks of incubation, the culture medium was removed and the cells were rinsed with PBS(-). The position of each colony was marked on the bottom plate of a dish with a marking pen. Next, 2 ml of 100 mM KPO₄ (pH 7.8), 5 mM ATP and 15 mM MgSO₄ containing 200 μ l of 10 mM luciferin was then added, and the dish was immediately put into the light shield chamber of the single-photon counting camera placed in a dark room for measurement of the luminescence. The light intensity was monitored for 1 hr and luminescent colonies were localized by their superimposition onto the image of the dish.

3. Results

3.1. 73-16 cell line containing a single *lox-neo* target

Plasmid pSF73 (Fig. 1A) was constructed by the insertion of a selectable marker, the 2.0-kb HSV-1 *tk* gene, into the *Bam*HI site of pSF1. pSF1 consists of both a *lox* site and a defective *neo* gene lacking a promoter and the first five codons. The *lox* site was designed to be in frame with the *neo* structural gene, but not to provide a translational start codon (ATG). After introduction of the defective *lox-neo* fusion gene into the host cell genome, the chromosomal *lox* site is targeted by Cre-mediated recombination using a trapping vector pSF86 (Fig. 1B). pSF86 provides an in-frame ATG and a strong promoter/enhancer sequence of the major immediate early promoter of human cytomegalovirus to regenerate a functional *neo* gene (Fig. 1C). The target *lox* site was placed into the *Ltk-* cell line by electroporation of pSF73 into the *Ltk-* cell line with subsequent selection of 36 independent TK⁺ colonies. Southern blot analysis identified nine of the 36 cell lines to be single-copy integrants and to contain an intact copy of the *lox-neo* target. In all of these cell lines, integration occurred in the 3.1-kb *Ap* region between the *lox* site and the carboxyl terminus of the *tk* gene (data not shown). One of the recipient cell lines, 73-16, was used to trap promoter sequences as described in this paper. The 73-16 cell line can be distributed by request.

3.2. Site-specific integration of pSF86 and its derivatives into 73-16 cells

To examine whether promoter sequences can in fact be trapped by Cre-*lox* site-specific recombination, we cloned the 648-bp *tk* promoter sequence into the *Bam*HI site of the pSF86 promoter-trapping vector to generate pSF86-*tk* and pSF86-*kt*, plasmids distinguished by *tk* promoter orientation: In pSF86-*tk*, the *tk* promoter is in the normal orientation, 5' to the luciferase gene, whereas in pSF86-*kt* the *tk* promoter is in the opposite orientation. For a negative control, a 610-bp DNA fragment from the *neo* gene

Table 1. Cre-mediated DNA targeting in cell line 73-16.

DNA	G418 ^R Colonies
pMAMneo-luc	5606
pSF86	0
pBS185	0
pSF86- <i>tk</i>	0
pSF86- <i>kt</i>	0
pSF86-610	0
pSF86 + pBS185	37
pSF86- <i>tk</i> + pBS185	16
pSF86- <i>kt</i> + pBS185	79
pSF86-610 + pBS185	18

Lipofection of 1×10^6 cells was with 2 μ g of the trapping vector pSF86 (or its derivatives), 0.5 μ g of the Cre expression vector pBS185, or with both plasmids. pMAMneo-luc contains an intact *neo* gene under the control of SV40 promoter.

was cloned into the *Bam*HI site in pSF86 to generate pSF86-610. pSF86 and its derivatives were transfected into the 73-16 cells with or without Cre expression vector pBS185 to assess whether these plasmids integrate in a site-specific manner at the chromosomal *lox* target. Because recombination regenerates a functional *neo* gene, site-specific integrants can be selected directly by resistance to G418. Table 1 shows the results of a representative targeting experiment using pSF86, pSF86 derivatives and pBS185. Neither the trapping vector nor the Cre expression vector alone generated G418-resistant colonies; however, co-transfection with both vectors generated a substantial number.

To confirm the occurrence of correct integration of the trapping vector at the chromosomal *lox* target site and to determine the number of copies integrated, three of each of the representative transformants were randomly selected and subjected to Southern blot analysis. Figure 1C shows the structure of the *lox-neo* region in the recipient cell and the structure anticipated after site-specific single-copy integration of pSF86. Genomic DNA digested with both *Eco*RI and *Xho* I was hybridized with a hCMV-specific probe. A 2.15-kb *Xho* I-*Eco*RI fragment in the recipient 73-16 cell is replaced with a novel 2.8-kb *Eco*RI fragment by the integration of pSF86 at the chromosomal *lox* site. Each G418^r transformant exhibited the presence of the 2.8-kb fragment consistent with site-specific integration at the chromosomal *lox* site (Fig. 2A). Six of the 12 transformants (pSF86 #3, pSF86-*tk* #1, #2, pSF86-*kt* #1, pSF86-610 #2, #3) hybridized with other bands in addition to the 2.8-kb fragment suggesting either tandem multiple integration events or illegitimate integration events as well as site-specific recombination. Genomic DNA was also digested with both *Eco*RV and *Sal*I, and subjected to Southern blot hybridization us-

ing a probe (1.4 kb) specific for the luciferase gene (Fig. 2B). This made it possible to analyze the structure of the region flanking the luciferase gene and the pSF86 cloning site. All transformants except pSF86 #3, pSF86-*tk* #1 and #2 showed hybridization signals of the expected size. pSF86 #3 was found to carry a deletion in the luciferase gene region. Integrants pSF86-*tk* #1 and #2 contained illegitimate recombination events in addition to site-specific integration.

3.3. Promoter activity of cloned DNA fragments

Extracts of the integrants were prepared and assayed for luciferase activity using Argus-50/CL as described in Materials and Methods. The luciferase activity of 10 μ l of extract was measured and compared in each transformant (Table 2). The parental cell line 73-16 showed 4.0 photon/min/ μ g protein which is nearly the same as the background noise of the Argus-50/CL. All of the pSF86 integrants show approximately the same level of luciferase activity (mean value of 4.3). Integrants of pSF86-610 used as a negative control are also uniform in gene expression and exhibit a mean luciferase activity of 4.1. Consequently, these integrants all showed essentially the background level of luciferase activity. In contrast, pSF86-*tk* integrants containing the *tk* promoter 5' to the luciferase gene in the normal orientation revealed 21- to 35-fold greater luciferase activity over that seen in 73-16. One of the pSF86-*tk* integrants, #2, showed evidence of a high level of illegitimate recombination and multiple copies of the luciferase gene in Southern blot analysis (Fig. 2). The luciferase activity of this integrant was the highest observed (141.3), in keeping with the Southern hybridization result. The luciferase activity of pSF86-*kt* integrants containing the *tk* promoter 5' to the luciferase gene in the reverse orientation was about twofold higher than that observed for 73-16. Among all integrants assayed, only the luciferase activities of the pSF86-*tk* integrants were directly detectable at the colony level. Figure 3 shows ~ 50 colonies of pSF86-*tk* integrant #3 on a 10-cm culture dish subjected to direct imaging of luciferase activity using the Argus-50/CL.

4. Discussion

We have developed a new system which traps DNA sequences containing promoter activity from a specific region of chromosome. The result of a model experiment using the *tk* promoter and the non-promoter sequence derived from the *neo* gene clearly demonstrated that our system is capable of efficiently trapping promoter sequences.

In the trapping vector pSF86, the SV40 poly(A) signal was inserted 5' upstream of the promoterless luciferase gene to repress illegitimate transcription from the neigh-

Table 2. Luciferase activity from stable transformants.

Trapping Plasmid	Transformant No.	Luciferase Activity (Photon/min/ μ g protein)
pSF86	1	4.6
	2	5.5
	3	2.9
pSF86- <i>tk</i>	1	84.0
	2	141.3
	3	117.7
pSF86- <i>kt</i>	1	8.9
	2	8.3
	3	10.0
pSF86-610	1	5.2
	2	4.6
	3	2.6

The results are the average of assays from two independently prepared extracts from each transformant. Background luciferase activity in the parental strain 73-16 was 4.0 photon/min/ μ g protein.

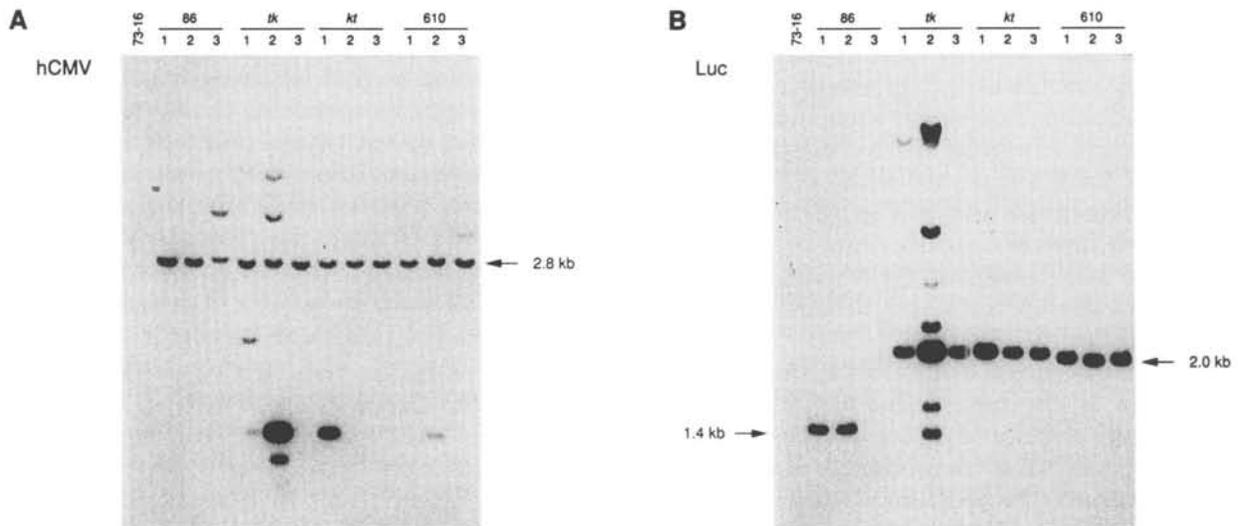


Figure 2. Site-specific integration of the trapping vector or its derivatives by Cre-catalyzed recombination. Genomic DNA (5 μ g) from the parental 73-16 cell and 12 independent Cre-mediated integrants was digested with both *Eco*RI and *Xho*I (A) or with both *Eco*RV and *Sal*I (B), and subjected to Southern blot hybridization analysis using either a CMV (A) or a luciferase gene probe (B). See Fig. 1C for the structure predicted after integration of pSF86.

boring DNA sequences. To examine the usefulness of this approach, plasmid pSF85, from which the SV40 poly(A) signal was removed, was transfected into the 73-16 cell line using the Cre expression plasmid pBS185. Three independent integrants were isolated, and the luciferase activities were assayed as before. The activities of the transformants were 34.4, 55.3, and 46.2. These values were 8- to 14-fold higher than those of the parental 73-16 cell. In contrast, the site-specific integrants from pSF86 showed luciferase activities of 4.6, 5.5, and 2.9, essentially the same level of activity as 73-16. Thus, insertion

of the SV40 poly(A) signal is essential to minimize the background luciferase activity.

In this report, lipofection was used as the method of DNA transfection. This has the advantage of reducing both the number of cells and amount of DNA needed for transfection as well as simplifying the experimental operation. Previously, site-specific integrants in mammalian cells using the Cre-*lox* system have been obtained by electroporation with at least 90% of the integrants being insertion of a simple single-copy of the *lox* targeting vector.^{12,13} Using lipofection, a tenfold increase in

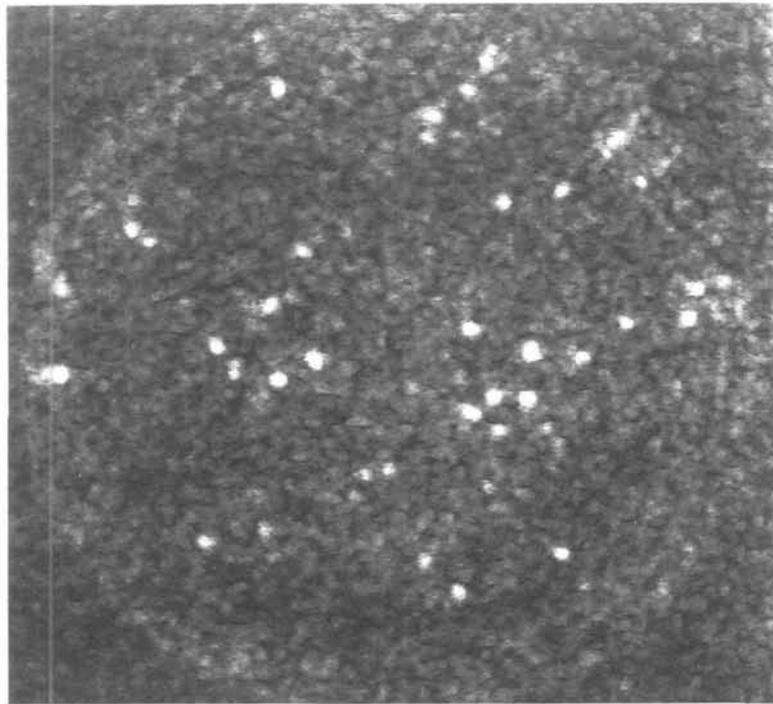


Figure 3. Direct detection of luciferase activity in colony level on a 10-cm culture dish. About 50 pSF86-*tk* transformant #3, a single-copy integrant (See Fig. 2), were plated on a 10-cm tissue culture dish and analyzed using the Argus-50/CL as described in Materials and Methods.

the number of integrants over that obtained with electroporation was achieved (data not shown). However, only 50% of integrants achieved by lipofection are simple site-specific integrants; the remaining 50% contain multiple tandem integration and/or illegitimate integration of vector sequences in addition to the site-specific integration events. These results suggest that DNA uptake into mammalian cells is much more efficient with lipofection than with electroporation. To moderate the high level of undesired recombination, it may be effective to reduce the amount of vector used in the transfection. Furthermore, because the activation of the luciferase gene by illegitimate recombination may increase the number of false-positive colonies, modification of DNA transfection as outlined above as well as insertion of stop codons 5' upstream of the luciferase gene by the addition of the SV40 poly(A) signal to the trapping vector may be necessary.

Another key issue in a successful promoter-trapping system is the selection of the appropriate endonuclease. Because, any given restriction enzyme may cleave a promoter sequence thereby precluding its identification, we have devised a system in which five different endonucleases may be used. A second consideration is the method used for the recovery of the trapped DNA fragments derived from the integrants. The use of PCR amplification to achieve this is frequently problematic, possibly

because of the GC-rich nature of many promoters. In these cases, the targeted construct can be directly excised from the genome as a circular molecule by reintroduction of pBS185. The resulting plasmid is transformed into *E. coli* and selected for ampicillin-resistance encoded by the β -lactamase gene in the vector. Another approach to this problem could be to use the *Not*I sites at both ends of the *Bam*HI cloning site. The molecules linearized with *Not*I are cloned into a vector containing an appropriate drug-resistance gene and the resulting plasmids are transformed into *E. coli*.

Our system was introduced into mouse *Ltk*- cells and, clearly, only promoter sequences which are functional in this cell line can be trapped. It is likely, based on the nature of *Ltk*- cells, that the majority of trapped sequences will be associated with the regulation of housekeeping genes. If, however, the system was used with ES cells, promoters of tissue-specific and developmental stage-specific genes could be isolated. *Cre-lox* site-specific recombination has already been successfully demonstrated in transgenic mice²¹⁻²³ as well as ES cells.^{24,25}

We believe that the isolation and analysis of novel promoter sequences will have a major impact. The collection of promoter sequences may give us an understanding of what kinds of transcription factors are functional in the same cell type. We would be able to address the question by analyzing the trapped sequences in this system.

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