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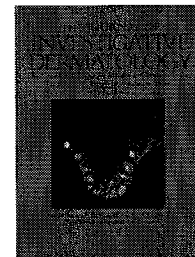

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Journal of Investigative Dermatology
Volume 112 Issue 5 Page 828 - May 1999
doi:10.1046/j.1523-1747.1999.00583.x

Promoter/Enhancer Cassettes for Keratinocyte Gene Therapy

To the Editor:

Successful keratinocyte gene therapy requires that transcriptional units express the therapeutic gene properly in keratinocytes *in vivo*. Promoter/enhancer cassettes containing these units should be examined under *in vivo* conditions (Cheng *et al.* 1993). In this study, we transferred plasmids that were constructed by introducing various promoter/enhancer cassettes fused with the *lacZ* (reporter) gene into keratinocytes by direct injection of naked DNA (Hengge *et al.* 1995), and evaluated the cassettes by measuring β-galactosidase (β-gal) expression.

Plasmid pNASSβ (CLONTECH, Palo Alto, CA) was a promoterless expression vector of the *lacZ* gene that contained a simian virus 40 (SV40) RNA splice site, which is an SV40 polyadenylation signal. For convenience, we refer to this plasmid as pZ(-). To synthesize the promoter segments of the mouse 230 kDa bullous pemphigoid antigen 1 gene, we carried out polymerase chain reaction (PCR) using a mouse bullous pemphigoid antigen 1 genomic clone as a template (Sawamura *et al.* 1994). The DNA segments extending from -1133 to -1, -525 to -1, and 213 to -1 were inserted into pZ(-) to produce p1.1 BPZ, p0.5 BPZ, and p0.2 BPZ, respectively. We synthesized the promoter segments of the human K5 and K10 genes by PCR using human genomic DNA as a template. The K5 and K10 DNA fragments that extended from -840 to -1 (Ohtuki *et al.* 1992) and from -1200 to -1 (Rieger & Franke 1988), respectively, were inserted into pZ(-) to produce pK5Z and pK10Z, respectively. All PCR products were sequenced and no PCR errors were detected from comparisons with the original sequences. Plasmids pAGS-lacZ and pCMS-lacZ contained the chicken modified β-actin promoter and 660 bp *HincII-HindIII* cytomegalovirus-immediate early (CMV-IE) enhancer/promoter, respectively (Miyazaki *et al.* 1989), and these two promoters were followed by *lacZ* and a SV40 polyadenylation signal. The *HincII-NcoI* region of the CMV-IE enhancer was introduced directly into the *XhoI* site, using a *SalI* linker, located the 5'-end of the β-actin promoter in pAGS-lacZ, and resulted in pCAGS-lacZ (Niwa *et al.* 1991). Plasmids pSV40S-lacZ and pK10S-lacZ were produced by replacing the *XhoI-HindIII* fragment of the β-actin promoter in pAGS-lacZ with the SV40 early promoter and the 1.2 kb K10 fragment, respectively. Plasmid pCAGGS-lacZ was constructed by replacing a SV40 polyadenylation signal with a 3'-flanking sequence of the rabbit β-globin gene, which included a polyadenylation signal (Niwa *et al.* 1991). Plasmid Rous sarcoma (pRS)-lacZ was constructed by replacing the *HindIII-BamHI*

Manuscript received June 17 1998; revised January 25 1999; accepted for publication February 2 1999.

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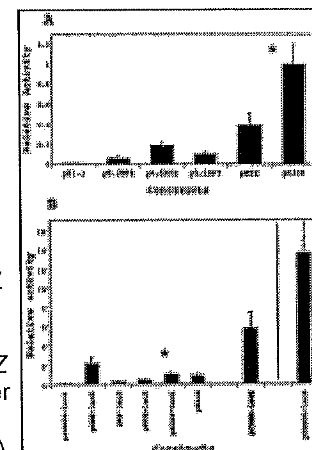

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Fig. 1 **Comparison of the β-gal activities in keratinocytes *in vivo* after transfer of various constructs....**

fragment, including the chloramphenicol acetyltransferase gene, of pRSV-cat (Gorman *et al.* 1982) with *lacZ*. Each plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients and two or three plasmid preparations were used for the experiments.

Hirosaki hairless rats were used for these experiments (Sawamura *et al.* 1997). The required plasmid was diluted with phosphate-buffered saline to produce a DNA concentration of 100 ng per μl, and 30 μl (total amount, 3 μg) was injected as superficially as possible into the subepidermal dermis using a 30-gauge needle. At various times after transfer, 6 mm punch biopsy specimens were obtained from the transfected area.

The β-gal activities in the rat keratinocytes were quantitated using our previous method (Sawamura *et al.* 1997), with certain modifications. Epidermal sheets were obtained from the 6 mm punch biopsy samples by 10 mg per ml dispase treatment (3 h at 37°C), and keratinocyte suspensions were obtained from these epidermal sheets by 0.25% trypsin treatment (30 min at 37°C). The keratinocytes were lyzed in 40 μl lysis buffer, centrifuged at 12,000 r.p.m. for 2 min, and a 5 μl aliquot of the resulting cell extract was assayed using the Luminescent β-Galactosidase Genetic Reporter System (CLONTECH) and a chemiluminometer to measure the chemiluminescence. The β-gal activity was expressed as light units per μg protein (Protein Assay Kit, BIO-RAD, Heracles, CA). Six separate samples from one experiment were subjected to this assay. We repeated the experiments at least three times to confirm their reproducibility.

Epidermal sheets from the injected area were obtained after dispase treatment, then digested with trypsin, washed three times extensively with phosphate-buffered saline, lyzed in 40 μl lysis buffer, and centrifuged for 2 min. A 2 μl aliquot of each cell extract was used as a template and then PCR was performed to measure the *lacZ* gene in the cells semiquantitatively. We used two primers, 5'-GACGATGGTGCAGGATATCC-3' and 5'-ACTGACGAAACGCCTGCCAG, to amplify the 567 bp *lacZ* segment that extended from 1430 to 1996 (MacGregor & Caskey 1989). The amount of each PCR product was estimated by 1.5% agarose gel electrophoresis and ethidium bromide staining. Before the experiment we established the PCR conditions that resulted in exponential amplification.

Plasmids p0.2 BPZ, p0.5 BPZ, p1.1 BPZ, pK5Z, pK10Z, and pZ(-) had identical plasmid backbones (**Fig. 1 *A***). Plasmids pSV40S-lacZ, pCMS-lacZ, pAGS-lacZ, pCAGS-lacZ, pK10S-lacZ, and pRS-lacZ shared a different plasmid backbone (**Fig. 1 *B***). The β-actin promoter with CMV-IE enhancer and the 3'-flanking sequence of the rabbit β-globin gene in pCAGGS-lacZ were also examined, although its backbone was different from those of the first two groups (**Fig. 1 *B***). The β-gal activity was assayed 24 h after transfer and the β-gal activities of these constructs were expressed relative to those of K10 constructs, pK10Z in **Fig. 1(*A***) and pK10S-lacZ in **Fig. 1(*B***).

Fig. 1 shows that the specific β-gal activities of these constructs varied. Of the promoters expressed virtually almost specifically in keratinocytes, the K10 promoter resulted in relatively high-level β-gal expression, whereas bullous pemphigoid antigen 1 promoter had a weak effect (**Fig. 1 *A***). Of the viral promoters, the CMV-IE promoter exhibited much higher activity than the SV40 promoter (**Fig. 1 *B***) and the RS virus long-terminal repeat. Because the CMV-IE promoter was strongest among the simple promoters that were excised from original genes, we examined two other constructs, pCAGS-lacZ and pCAGGS-lacZ, to determine the effects of the CMV-IE enhancer and the 3'-flanking sequence of the rabbit β-globin gene on the transcriptional activity of keratinocytes. The activity of pCAGS-lacZ was about 15 times greater than that of pAGS-lacZ and that of pCAGGS was about two and six times those of pCAGS-lacZ and pCMS-lacZ, respectively (**Fig. 1 *B***).

The amount of transgene was measured semiquantitatively 24 h after the introduction of various constructs. We introduced pAGS-lacZ, pCAGS-lacZ, pCAGGS-lacZ, and pCMS-lacZ, and performed PCR amplification of the lacZ gene. We also injected phosphate-buffered saline as a control. The result showed no

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Fig. 2 **The amount of transgene in keratinocytes after gene transfer.** The amount of transgene was measu...

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(1999)
Promoter/Enhancer Cassettes for Keratinocyte Gene Therapy.
Journal of Investigative Dermatology **112** (5), 828-830.
doi: 10.1046/
j.1523-1747.1999.00583.x

clear differences among the band intensities of the PCR products (**Fig. 2** ***A***), and the amounts of the transgene (*lacZ*) in the keratinocytes into which these plasmids introduced could not reflect a difference among the activities of the plasmids. We detected no bands in the sample of phosphate-buffered saline. We sequenced the PCR products and digested with appropriate restriction enzymes to test for PCR specificity (data not shown).

Cheng *et al.* (1993) carried out similar experiments using particle bombardment to introduce genes, and showed that the CMV-IE promoter was very active in keratinocytes. We also found that the CMV-IE promoter in pCMS-lacZ exhibited very high activity (**Fig. 1**). We also evaluated the activities of the promoter from genes that were specifically expressed in keratinocytes and found that the K10 promoter in pK10Z showed the highest activity (**Fig. 1** ***A***); however, the K10 promoter in pK10S-lacZ was still weaker than the CMV-IE promoter in pCMS-lacZ. Our results demonstrate that the CMV-IE promoter was superior to the other simple promoters, with respect to the activity level. In an attempt to take advantage of the highly active viral CMV-IE promoter, we assayed the activity of pCAGS. This construct was tested using several types of cell in culture; enhancement of expression was observed in L and CHO cells, but not in F9 cells (Niwa *et al.* 1991). The addition of CMV-IE enhancer increased β-gal expression in keratinocytes *in vivo* 15-fold. The 3'-flanking sequence of the rabbit β-globin gene was used for the construction of expression vectors in several groups and these vectors exhibited high levels of production of the inserted gene in various cells (Niwa *et al.* 1991; Karasuyama & Melchers 1988). We also tested the activity of pCAGGS-lacZ, in which 3'-flanking sequence of the rabbit β-globin gene was introduced into the 3'-end of the lacZ gene, resulting in a further 2-fold enhancement. We could not make precise comparisons among pCAGGS-lacZ and the other constructs because of different backbones, but pCAGGS-lacZ exhibited the highest activity for all the constructs tested. We constructed an interleukin-10 expression vector using this cassette, introduced it into keratinocytes *in vivo* by direct injection of naked DNA, and achieved systemic expression of transgenic interleukin-10 (Meng *et al.* 1998). This study has provided useful information for developing potential promoter/enhancer cassettes for keratinocyte gene therapy using the direct injection of naked DNA method.

This work was supported in part by a grant from the Ministry of Education, Japan. We are grateful to Ms. Yoko Uno and Ms. Komaki Hanada for their excellent technical assistance.

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Abbreviations

Go to: Choose Go

β-gal β-galactosidase
CMV cytomegalovirus
IE immediate early
RS Rous sarcoma
SV40 simian virus 40

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