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Short communication

Comparison of hCMV immediate early and CaMV 35S promoters in both plant and human cells

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Abstract

Cauliflower mosaic virus 35S promoter, widely used in transgenic crop plants, is known to be recognized in widely differing kinds of cells. Its activity in human cells may have impact on the risk assessment for the environmental release of genetically modified plants. In this study, transient expression of several constructs containing β-glucuronidase (GUS) gene driven by cauliflower mosaic virus 35S promoter or by immediate early promoter of human cytomegalovirus (pCMV) was tested in both potato leaf protoplasts and cultured human cells. The results showed very low but measurable activity of 35S promoter in human 293T-cells (0.01% of that revealed when using pCMV) and in 293 cells that do not produce SV40 T antigen this activity was even lower. On the other hand, in potato protoplasts, pCMV displayed nearly 1% activity seen with p35S.
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1. Introduction

The strong cauliflower mosaic virus (CaMV) 35S promoter (p35S) is used for the expression of transgenes in nearly all genetically modified crop plants. The promoter functions efficiently in all plants, as well as in green algae, yeast, and *Escherichia coli* (Assaad and Singer, 1990; Pobjecky et al., 1990). Hot discussions are under way in the scientific community about its possible activity in animal cells in association with safety concerns in genetically modified plants. Nevertheless, few relevant data have been published as yet: Ballas et al. (1989) have shown that p35S supported high level of reporter gene expression in mature *Xenopus* oocytes. The CaMV promoter has worked at least as well as the SV40 promoter in this system. In extracts of HeLa cell nuclei, p35S has been more efficient than the adenovirus 2 late promoter (Burke et al., 1990). Of course, these

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systems provide very anomalous transcription conditions and for the typical animal cells the results are probably irrelevant.

In this study, we compared the transient expression of bacterial β-glucuronidase (GUS) driven by p35S in both plant and human cells. To evaluate potency of p35S in human cells, p35S was exchanged with immediate early promoter of human cytomegalovirus (pCMV) in several constructs, and such cassettes were used as expression standards in human cells. pCMV is a very strong promoter that is often utilized in mammalian expression vectors (Boshart et al., 1985).

Besides comparison of p35S and pCMV in human cells, the constructs with both promoters were also simultaneously tested in plant protoplasts.

2. Materials and methods

2.1. DNA constructs

"MonoGUS" expression cassette containing CaMV 35S promoter and the first 15 codons of CaMV ORF I fused with the GUS gene (Bonneville et al., 1989) was used as standard in all experiments. Signal for transcription termination was derived from CaMV, too (Fig. 1). This expression cassette in pUC plasmid was modified using procedures for the preparation of recombinant DNA plasmids described by Sambrook et al. (1989). Human cytomegalovirus promoter (pCMV) was excised from the vector pBSC (Šmahel et al., 2001) with enzymes *Xba*I and *Sal*I, cloned into pUC18 and recloned so that it was flanked by *Kpn*I sites. *Kpn*I fragment with pCMV was inserted into *Kpn*I site of "MonoGUS", 9-bp downstream of the transcription start of CaMV p35S, to obtain plasmid pCB0182 with both promoters in tandem and set of sites *Kpn*I, *Sma*I, *Bam*HI and *Xba*I in inverted orientation flanking the pCMV (Fig. 1). CaMV p35S promoter was then excised by digestion with *Bsp*MII site in front of p35S and partial digestion with *Xma*I in the polylinker and religation. Resulting plasmid pCB0199 has CaMV p35S replaced by pCMV in the MonoGUS cassette.

For comparison, N-terminal ORF I codons of the ORF I/GUS fusion protein were excised from the pCB0182 together with pCMV using digestion with *Bam*HI in the linkers and religation to obtain pCB1116-"MonoGUS" without ORF I fusion. Because of partial *Bam*HI digestion, pCB0192 was obtained, too, with the quasi-original MonoGUS structure. The only difference is the palindromic set of sites *Kpn*I, *Sma*I, *Bam*HI, *Sma*I, *Kpn*I 9-bp downstream the CaMV p35S transcription start. Similarly, CaMV polyadenylation sequence was excised with the enzyme *Sac*I (pCB1161; Fig. 1).

All plasmids were isolated by the "cleared lysate method" and purified by cesium chloride/ethidium bromide centrifugation (Sambrook et al., 1989). They were dissolved in water and adjusted to 2 μg DNA μl^{-1} before transfection.

2.2. Potato protoplast transfection and GUS activity assay

Protoplasts were isolated from the leaf mesophyll cells of *Solanum tuberosum* L. cv. Bintje according to Bříza and Machová (1991). 0.5×10^6 of freshly prepared protoplasts in 1 ml SW_{11} culture medium (Bříza and Machová, 1991) were PEG transfected (Negrutiu et al., 1987) with 50 μg of pUC plasmid containing GUS expression cassette. Protoplasts were incubated for 24 h, collected, and lysed in 50 μl of GUS buffer (Jefferson, 1987). GUS activity as well as the protein content were measured in 10 μl of homogenate; the typical protein content was 0.5–1.2 μg μl^{-1}. GUS activity was assayed with the 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescent substrate according to Jefferson (1987) and proteins were determined according to Bradford (1976).

2.3. Human cell transfection and GUS activity assay

293 human embryonal kidney cells (Graham et al., 1977) or 293T-cells derived from 293 cells by transduction with the gene coding for simian virus 40 (SV40) large T antigen (DuBridge et al., 1987) were used.

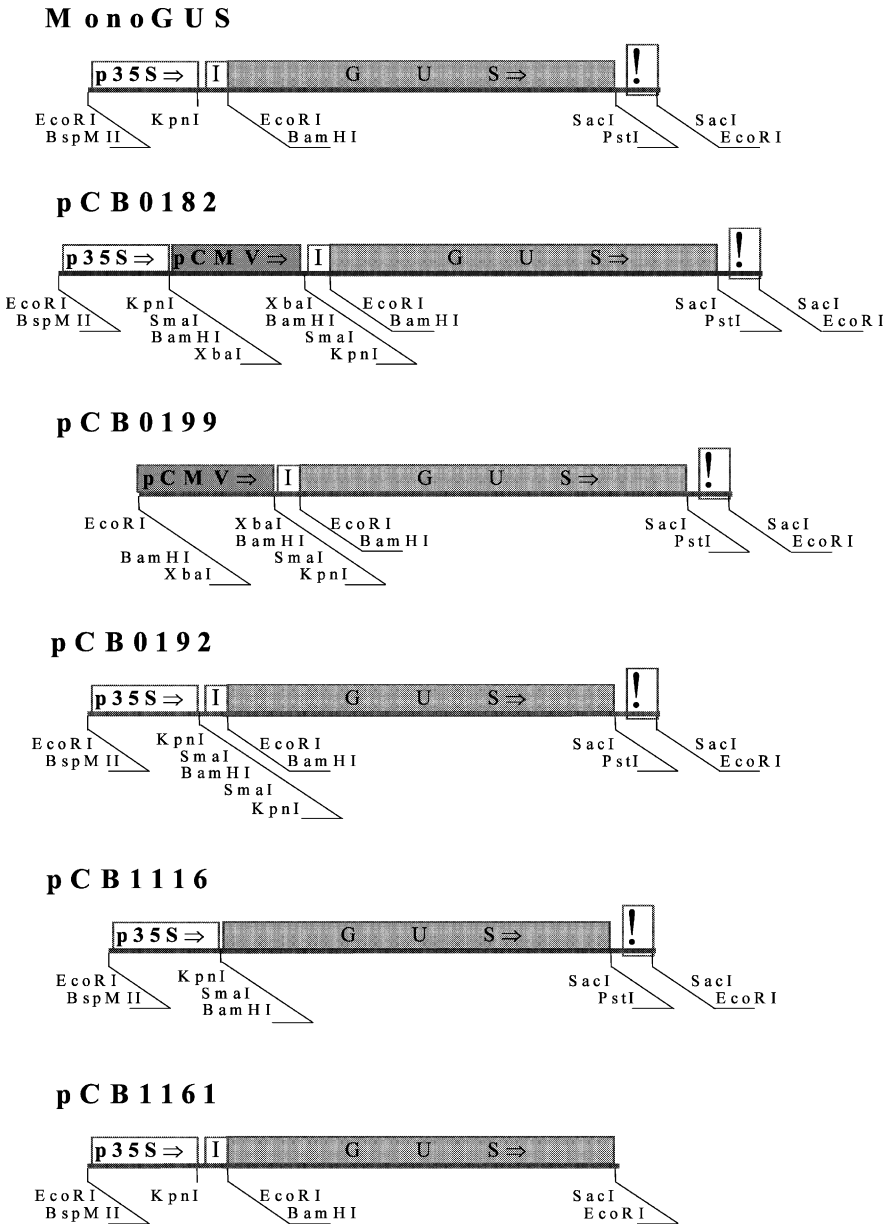

Fig. 1. Structure of the expression cassettes in pUC plasmids used for transfection. The bars represent segments of DNA that have been inserted into plasmid vectors. The same symbols are used as in Table 1. Only relevant restriction sites are shown.

0.7×10^6 of cells were seeded into 6-cm plates and transfected on the next day by modified calcium phosphate precipitation in HEPES-buffered saline solution (Kingston et al., 1997) with 6 μg of plasmids. Cells were incubated for 48 h, collected, and lysed in 200 μl of GUS buffer. GUS

activity and protein concentration were measured as described for potato protoplasts. The typical protein content was 0.7–1.3 μg μl^{-1}.

3. Results

The results of GUS assays are summarized in Table 1. CaMV p35S promoter conferred very low expression in human 293T-cells in all cassettes studied (about 10,000 times lower than pCMV). On the other hand, pCMV showed surprisingly high activity in plant cells—only about 100 times lower than the extremely strong plant p35S promoter. To the best of our knowledge, this observation has not yet been reported.

When pCMV was inserted between p35S and I/GUS gene (pCB0182), the GUS activity was substantially reduced in plant protoplasts. In 293T-cells, the expression of GUS from the tandem of p35S-pCMV promoters was comparable with that from pCMV only.

N-terminal ORF I codons from the natural CaMV 35S mRNA provide the optimal sequence for the eucaryotic translation start resulting in very high level of GUS expression in plant protoplasts (Bonneville et al., 1989) as well as in transgenic plants (Vlasák and Toušová, 1997). Deletion of the ORF I codons (pCB 1116) that reveals the original start codon of bacterial β-glucuronidase as the first ATG on the mRNA causes drop of GUS activity in potato protoplasts to about 6% and the deletion of the CaMV transcription termination signal (pCB 1161) had even stronger effect. All these effects could also be seen in human cells, but a more exact evaluation was impossible because of low GUS activity. The results are in accordance with the published data describing eucaryotic translation start consensus sequence (Kozak, 1981) and mRNA stability improvement by appropriate polyadenylation sequence. Nevertheless, the most pronounced drop of activity occurred when using structure where symmetrical (palindromic) linker *Kpn*I–*Sma*I–*Bam*HI–*Sma*I–*Kpn*I was obtained after several cloning steps 9-bp downstream the mRNA cap (pCB0192). This hairpin structure probably impairs ribosome landing on mRNA substantially. After excision of the palindrom with enzyme *Kpn*I, we obtained plasmid pCB1117 with the same structure and activity as "MonoGUS" (not shown in Table 1).

Human 293T-cells express T antigen of SV40 that has been shown to transactivate some viral and cellular promoters (Rice and Cole, 1993). To assess the influence of T antigen on the activity of p35S and pCMV promoters, GUS activity was also determined in 293 cells lacking T antigen. In accordance with published data (Moens et al.,

Table 1
Expression of GUS in potato and human cells driven by CaMV 35S or hCMV promoters

Expression cassette	Structure	Relative activity (%)	
		Potato protoplasts	293T-cells
MonoGUS	p35S-I/GUS!	**100**	0.012 ± 0.002
pCB 0199	pCMV-I/GUS!	0.61 ± 0.16	**100**
pCB 0182	p35S-pCMV-I/GUS!	0.73 ± 0.02	106 ± 4
pCB 1116	p35S-GUS!	6.5 ± 1.2	0.005 ± 0.004
pCB 1161	p35S-I/GUS	5.0 ± 0.3	0.001 ± 0.001
pCB 0192	p35S-MCS-I/GUS!	1.50 ± 0.14	0.007 ± 0.001

Each value represents the mean of three repeated experiments ± standard deviation. "MonoGUS" cassette was always used as a standard. Its absolute activity varied in potato protoplasts in the range 210–920 pmol MUG min^{-1} μg $protein^{-1}$ and the actual value was used as 100% in each experiment. In human cells, the range for MonoGUS was 0.14–0.33 pmol MUG min^{-1} μg $protein^{-1}$. pCB0199 with the same fusion driven by hCMV promoter was used as a 100% standard in experiments with human cells. The activities determined varied in the range 1100–2420 pmol MUG min^{-1} μg $protein^{-1}$. The background increase of fluorescence represented maximally 0.1 pmol MUG min^{-1}. Symbols: p35S: CaMV 35S promoter; pCMV: hCMV immediate early promoter; !: CaMV transcription termination signal, I: first 15 codons of CaMV ORF I; MCS: multiple cloning site-sequence with restriction sites *Kpn*I–*Sma*I–*Bam*HI–*Sma*I–*Kpn*I.

2001), these experiments demonstrated profound effect of T antigen on the expression driven by pCMV (GUS activity was 10–20 times lower in 293 cells—data not shown). Activity of p35S was reduced similarly in the absence of T antigen.

4. Discussion

GUS expression driven by p35S promoter could be readily detected in 293T human cells, although in a much lower extent than in plant protoplasts. Nevertheless, such direct comparison is of little value because of very different conditions for DNA transfection in different cell types. Comparison of CaMV 35S (plant) and hCMV (animal) promoters in each type of cells (Table 1) shows several thousand times lower activity of p35S in human cells. It indicates that the potential hazards associated with the use of p35S may not be so serious as it is sometimes maintained. Recent reports have suggested that because of a proposed 'recombination hotspot', the consumption of transgenic plants that contain the 35S promoter may result in 'inappropriate overexpression of genes' leading to cancer in humans, or that recombination may lead to the reactivation of 'dormant viruses' or the creation of 'new viruses' (Ho et al., 1999). It is questionable whether relatively low transcription activity of CaMV 35S promoter can induce such hazardous events in mammalian cells.

On the other hand, only about 100-fold lower expression of widely used animal pCMV in plant cells is surprising. Expression levels this high have not even been reported with some genuine plant promoters. This fact should be kept in mind in the course of design and use of eucaryotic expression vectors.

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