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ORIGINAL PAPER

Marit R. Myhre · Kristin A. Fenton · Julia Eggert · Kaare M. Nielsen · Terje Traavik

The 35S CaMV plant virus promoter is active in human enterocyte-like cells

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Abstract The 35S cauliflower mosaic virus (CaMV) promoter is commonly used to drive transgene expression in the genetically engineered (GE) crop plants that have been commercialized so far. Whether, and how far, the 35S promoter might be active in mammalian cells has been scientifically unsettled and controversial. Very recently it was established that the 35S promoter is transcriptionally active following transient reporter gene transfections in continuous cell lines of human [J Biotechnol 103:197–202, 2003] and hamster ovary [Environ Biosafety Res 3:41–47, 2004] fibroblasts. The initial exposure of a human organism to DNA from GE food takes place in the gastrointestinal tract (GIT). Hence, we have now investigated the promoter capacity of 35S in human enterocyte-like cells. We constructed expression vectors with 35S promoter inserted in front of two reporter genes encoding firefly luciferase and green fluorescent protein (GFP), respectively, and performed transient transfection experiments in the human enterocyte-like cell line Caco-2. It was demonstrated that the 35S CaMV promoter was able to drive the expression of both reporter genes to significant levels, although the protein expression levels might seem modest compared to those obtained with the strong promoters derived from human cytomegalo virus (hCMV) and simian virus 40 (SV40). Furthermore, computer-based searches of the 35S CaMV DNA sequence for putative mammalian transcription factor binding motifs gave a high number of hits. Some of the identified motifs indicate that transcriptional activation by the 35S CaMV promoter may be stronger in other human and animal cell types than in those investigated so far.

Keywords 35S Cauliflower mosaic virus promoter · Caco-2 cells · Green fluorescent protein · Luciferase · Transfection

T. Traavik (✉)
GENOK-Norwegian Institute of Gene Ecology, Science Park, N-9294 Tromsø, Norway
e-mail: terjet@genok.org
Tel.: +47-77-64-43-79/95-81-75-37
Fax: +47-77-64-61-00

M. R. Myhre · T. Traavik
Department of Microbiology and Virology, Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway

J. Eggert · K. M. Nielsen
Department of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway

M. R. Myhre · K. A. Fenton
Both authors have contributed equally, University of Tromsø, N-9037 Tromsø, Norway

Introduction

Cauliflower mosaic virus (CaMV) is a DNA-containing para-retrovirus replicating by means of reverse transcription [1]. One of the viral promoters, called 35S, is a general, strong plant promoter. It has been used to secure expression of the transgene in most of the genetically engineered (GE) crop plants commercialized so far. The 35S promoter is generally considered to be a strong constitutive promoter [2, 3], and it drives high levels of RNA production in a wide variety of plants, including plants well outside the host range of the virus [4].

Claims that the 35S promoter would not be active in mammalian cells [5] were never supported by any experimental data, on the contrary, indications that these assumptions might be incorrect had been published. Besides studies in *E. coli* [6] and in different types of yeasts, *Saccharomyces cerevisae* [7, 8], *Schizosaccharomyces pombe* [9, 10], there are also reports indicating that the 35S CaMV promoter might have potential for transcriptional activation in mammalian systems [11, 12]. Recently, more direct evidence on the basis of transient 35S-driven reporter gene experiments in mammalian cell lines was presented [13, 14]. There are distinct discrepancies between the results obtained in human embryonic kidney cells (293 and 293-T) found by Vlasak et al., and those obtained in Chinese hamster ovary cells (CHO) found by Tepfer et al. The former reported a very low specific activity of a 35S-driven *gus* (β-glucuronidase) gene, while the latter demonstrated a

considerable level of expression by a 35S-driven *luc* (firefly luciferase) gene construct. The divergent levels of expression may be due to the differences in experimental design, gene constructs, and cell cultures. The common, phenomenological conclusions, however, were that the 35S CaMV promoter was active in the recipient mammalian cell cultures employed, and that this may indicate a risk issue in relation to food and feed derived from transgenic plants, provided that plant DNA fragments are taken up from mammalian alimentary tracts [14, 15].

Uptake of food-derived DNA fragments from the intestines into the blood stream and some organs has been demonstrated in various animal species [11, 16–21] and recently also in humans [22]. The biological significance of such incidents is unclear. Whether and to what extent foreign DNA fragments may be transported to cell nuclei, become integrated into recipient genomes, or are actively transcribed in mammalian organisms is not well understood.

Provided that fragments containing intact 35S/transgene combinations are taken up by enterocytes and/or cells in the Peyer's patches [15, 23], it is not an unreasonable scenario that the transgene polypeptide product may be expressed in recipient cell nuclei even from an episomal location, if the 35S promoter is active in the particular host cell type. Thus, for experimental purposes, careful consideration of the origin and characteristics of candidate cell lines is crucial for the conclusions that can be drawn with regard to the potential of 35S promoter to drive the expression of transgenes in relevant human cells, e.g. the cells lining the intestinal wall.

In this study, we constructed novel vectors to examine to which extent the 35S CaMV promoter is able to mediate transient expression of the reporter genes *gfp* and *luc* encoding green fluorescent protein (GFP) and firefly luciferase (LUC), respectively, in the human cell line Caco-2, which shares a number of characteristics with human enterocytes [24–26]. We demonstrated that the 35S CaMV promoter is actively transcribing both reporter genes in these cells, although at considerably lower levels than the strong mammalian promoters from human cytomegalovirus (hCMV) and simian virus 40 (SV40).

Material and methods

Human cell lines

The human epithelial cell line Caco-2 was obtained from The European Collection of Cell Cultures (ECACC 86010202). Cells grown in 75 cm^2 flasks (NUNC, Rochester, NY) were passaged weekly at a split ratio of 1:4 using 0.05% trypsin in PBS with 0.022% EDTA. Cells at passages 1–30 were cultured in Eagle's Minimum Essential medium with Earle's BSS (EMEM) containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, supplemented with 20% fetal bovine serum (FBS) (all from Gibco, Paisley, Scotland). The cells were grown at 37 °C with a 5% CO_2 in air atmosphere. The cell line is isolated from a primary, colorectal adenocarcinoma, and upon reaching

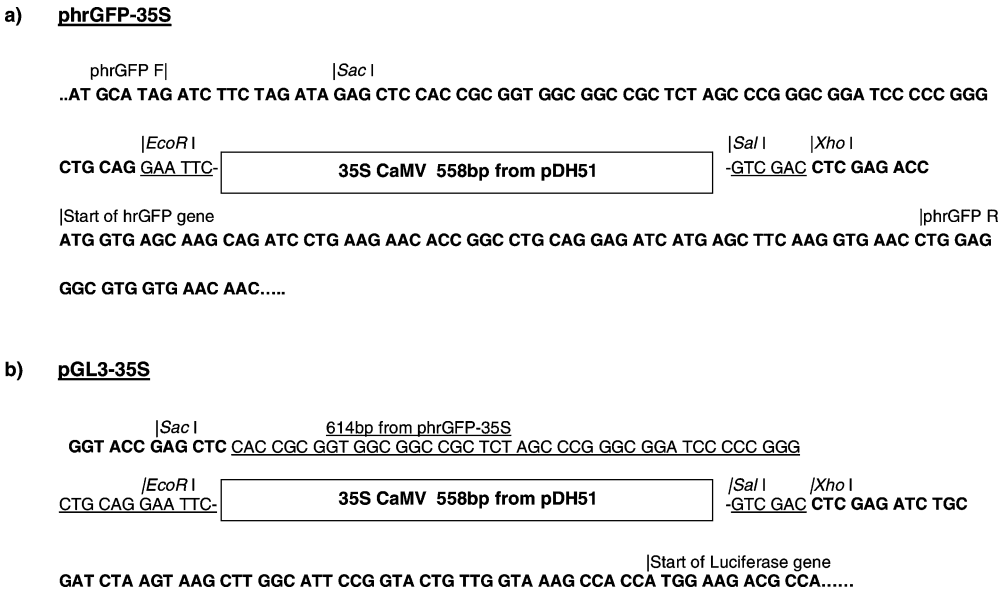

Fig. 1 Physical maps of the *gfp* and *luc* reporter gene expression plasmids used in this study. **(a)** Map of phrGFP-35S with the position of the 35S CaMV promoter inserted in front of the *GFP* reporter gene in the phrGFP plasmid. Only restriction sites relevant for subcloning the 35S CaMV sequence are depicted. The map includes localization of the primers used to sequence the inserted CaMV sequence and primers used to amplify a 184 bp sequence of the phrGFP-35S plasmid. **(b)** Map of pGL3-35S with the position of the 35S CaMV promoter inserted in front of the *Luciferase* gene in the pGL3-Basic plasmid. Additional plasmids were used in this study that were either promoter free, or contained the hCMV in front of the *GFP* gene, or the SV40 promoter in front of the *Luciferase* gene

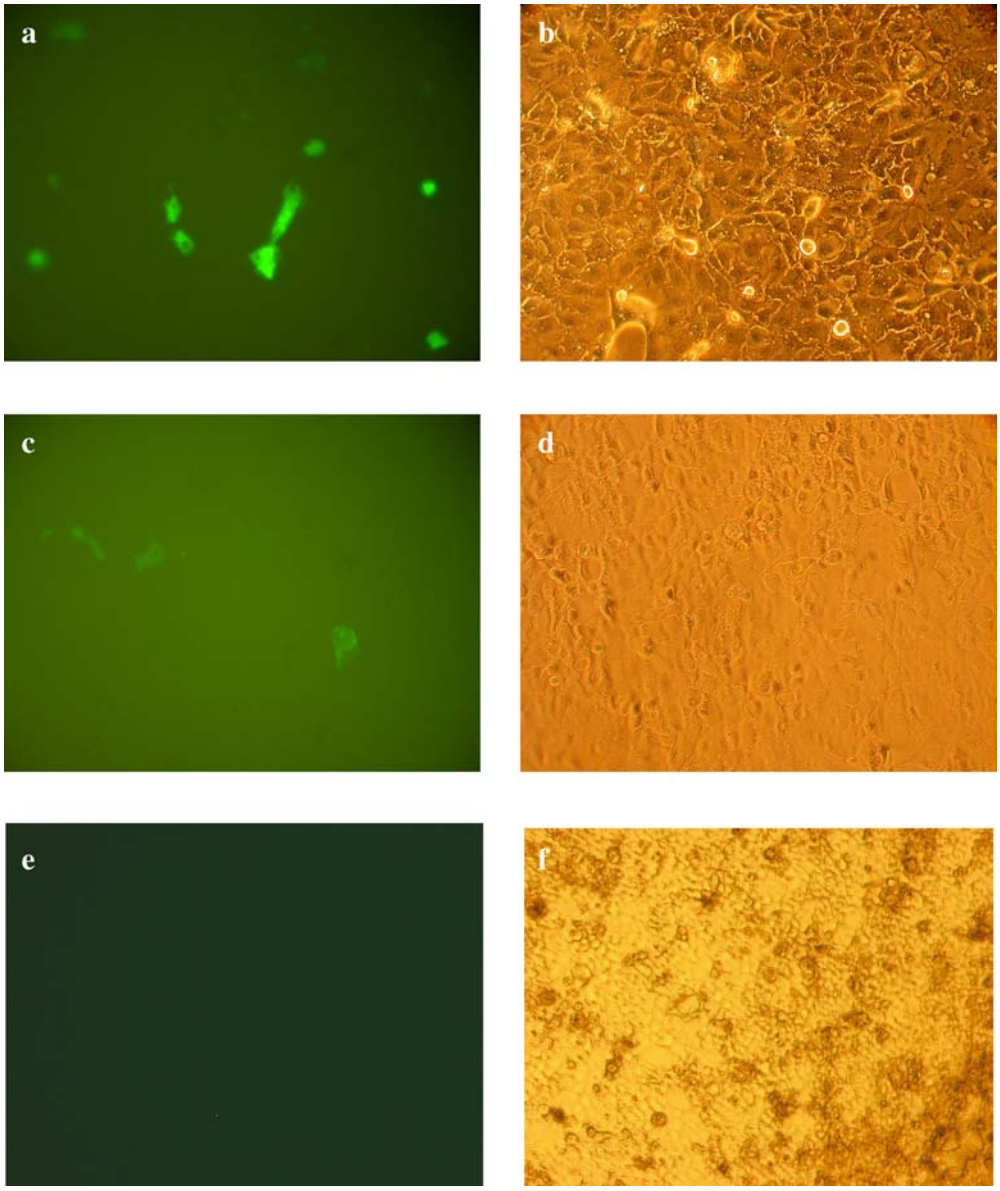

Fig. 2 Fluorescence microscopy of GFP expression in transfected Caco-2 cell cultures. Photographs of Caco-2 cells transfected with the plasmids phrGFP-hCMV **(a, b)** or phrGFP-35S **(c, d)**, both showing GFP expression. **(e, f)**: Caco-2 cells transfected with the promoterless plasmid phrGFP showing no sign of GFP expression. GFP expression was observed in a fluorescent microscope 48 h after transfection. **(a)**, **(c)**, and **(e)** show photomicrographs obtained by the fluorescence microscope; **(b)**, **(d)**, and **(f)** show the same cell cultures as they appear by light microscopy

confluence, the cells express characteristics of enterocytic differentiation.

Construction of plasmids

The 35S CaMV promoter obtained from pDH51 [27] (DSMZ, Braunschweig, Germany) was inserted in front of the *hrGFP* gene in the plasmid phrGFP (Stratagene, La Jolla, USA) as follows: The forward and backward primers were 5′-CGTATGTTGTGTGGAATTGTGAGC-3′ and 5′-CGATTAAGTTGGGTAACGCCAGG-3′, respectively; they were commercially synthesized (Invitrogen, Oslo, Norway). PCR was performed with Taq polymerase (Promega, Madison, USA) in a thermal cycler (Eppendorf, Hamburg, Germany) for 25 cycles at 94 °C, 30 s; 65 °C, 30 s; and 68 °C, 10 min. The size of the PCR product was verified by 1.0% agarose gel electrophoresis. The 879 bp product containing the 35S CaMV promoter, a multicloning site, and a termination polyadenylation (polyA) signal for the CaMV promoter from the pDH51 plasmid, was digested with the restriction enzymes *EcoRI* and *Sal I* to give a 558 bp 35S CaMV promoter containing fragment, a 226 bp CaMV polyA sequence and two 47 and 48 bp end products. The 558 bp fragment was excised from an agarose gel and purified using a gel-purifying column

(Qiagen, Hilden, Germany). The phrGFP vector was restriction digested with *EcoRI* and *Sal I*, and the PCR product was ligated into the vector creating the phrGFP-35S plasmid (Fig. 1a). The insert was sequenced directly using phrGFP F (5′-CCG TAT TAC CGC CAT GCA TAG-3′) and R (5′-GTT GTT CAC CAC GCC CTC CAG-3′) primers. Sequencing of either purified or cloned PCR products was performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, UK). Sequencing reactions were analyzed on a ABI 377 Sequencer (Perkin-Elmer, Boston, USA). The sequences were compared against the GenBank database using the program BLAST (National Center for Biotechnical Information, Bethesda, USA).

The commercial pGL3-Basic vector (Promega, Madison, USA) was used to create a plasmid with the *Luciferase* gene behind the 35S CaMV promoter: pGL3-35S. The phrGFP-35S plasmid was restriction digested with *SacI* and *XhoI* and the resulting 35S CaMV promoter fragment was ligated into the pGL3-Basic vector creating the pGL3-35S plasmid (Fig. 1b).

Transfection of Caco-2 cultures

Luciferase

Approximately 2.7×10^4 Caco-2 cells were seeded in 0.5 ml of growth medium (EMEM/20% FBS) per well in a 24-well plate (Gibco, Paisley, Scotland) and incubated at 37 °C in 5% CO_2 for 24 h to obtain 80% confluence. Transfection took place according to the manufacturer's protocol (Invitrogen, Oslo, Norway) using 0.4 μg plasmid DNA, 4 μl Plus reagent, and 1.5 μl Lipofectamine reagent for each well (24-wells tray). The mixture was added to the wells and incubated at 37 °C in 5% CO_2 for 3 h. The transfection medium was replaced with growth medium supplemented with 20% FBS and the cells grown for 48 h before analysis of Luciferase expression. The positive and negative control plasmids used for the Luciferase assay were pGL3-Control with the SV40 promoter in front of the *Luciferase* gene (pGL3-SV40) and the pGL3-Basic without a promoter in front of the *Luciferace* gene.

GFP

Caco-2 cells (1.5×10^5 cells) were seeded out in 2.5 ml of growth medium (EMEM/20% FBS) per well in a 6-well plate (NUNC, Rochester, USA) and incubated at 37 °C in 5% CO_2 for 24 h to obtain 80% confluence. Tranfection was carried out as above with 2 μg plasmid DNA, 32 μl Plus reagent, and 7.5 μl Lipofectamine reagent per well for a 6-well tray. The transfection mixture was incubated as above, and GFP expression was analyzed after 48 h. As positive and negative controls, we used the plasmid with the CMV promoter in front of the *GFP* gene (phrGFP-hCMV) and a plasmid without promoter in front of the *GFP* gene (phrGFP).

DNA isolation, Southern blotting, and Alu-PCR

Total DNA was isolated from the transfected cell cultures with Qiagen DNeasy tissue isolation Kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany). DNA was run on a 1% agarose gel and blotted to a Nytran N membrane (Schleicher and Schuell, Dassel, Germany) with a Turboblotter as recommended by the manufacturer. The membrane was hybridized at 68 °C for 3 h with a Digoxigenin (DIG)-labelled DNA probe constructed with the phrGFP-35S plasmid DNA as a template for the DIG High prime kit from Roche (La Roche Ltd, Basel, Switzerland). Alu-PCR was employed using specific primers for the human Alu sequence and the *hrGFP* gene to facilitate the possible detection of single integrations of the *hrGFP* gene in

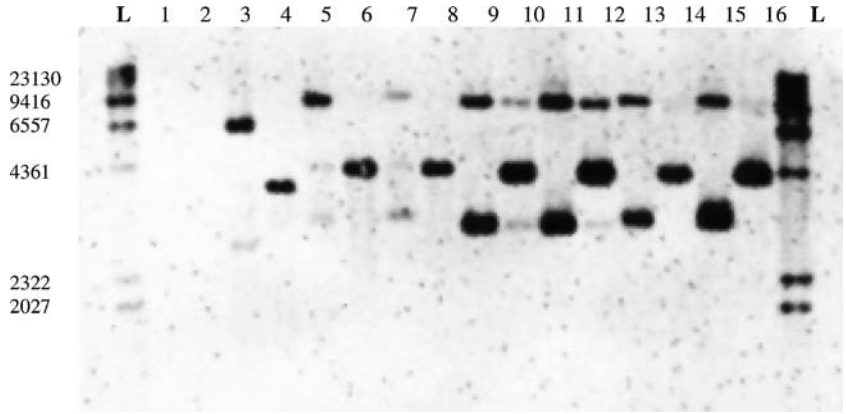

Fig. 3 Total genomic DNA of transfected Caco-2 cells hybridized against a DIG-labelled phrGFP-35S probe. Southern blot analysis of DNA extracted from mock-transfected Caco-2 cells (lane 1: uncut, lane 2: *EcoRI* cut), Caco-2 cells transfected with phrGFP (promoterless) (lane 3: uncut, lane 4: *EcoRI* cut), phrGFP-35S (lane 5: uncut, lane 6: *EcoRI* cut), and phrGFP-hCMV (lane 7: uncut, lane 8: *EcoRI* cut) constructs. Positive control phrGFP-CaMV 25 ng (lane 9: uncut, lane 10: *EcoRI* cut), phrGFP-CaMV 50 ng (lane 11: uncut, lane 12: *EcoRI* cut), phrGFP-hCMV 25 ng (lane 13: uncut, lane 14: *EcoRI* cut), and phrGFP-hCMV 50 ng (lane 15: uncut, lane 16: *EcoRI* cut) constructs. L, DIG marked ladder. The membrane was hybridized with a probe generated by random labeling of the phrGFP-35S plasmid

the cellular chromosomes [28, 29]. A human consensus Alu sequence [30] was used to design suitable primers, namely Alu 1F (5′-TCCCAGCTACTCGGGAGGCTGAGG-3′, nt 164–187 from the consensus sequence) and Alu 2R antisense (5′-GCCTCCCAAAGTGCTGGGATTACAG-3′, nt 47–23 from the consensus sequence) [28, 31]. The forward and reverse primers x-35sGFP-2F (5′-CAACCACGTCTTCAAAGCAA-3′) and x-GFP35s-2R (5′-AGGATCTGCTTGCTCACCAT-3′), respectively were used to amplify a 184 bp sequence from the 35S CaMV promoter sequence. For the Alu amplification, PCR was performed with Taq polymerase (Promega, Madison, WI) in a thermal cycler (Eppendorf, Hamburg, Germany) for 35 cycles at 95 °C, 1 min; 60 °C, 1 min; and 72 °C, 5 min. For the 35S CaMV sequence amplification, PCR was performed for 30 cycles at 94 °C, 1 min; 55 °C, 30 s; 72 °C, 30 s. PCRs using combinations of the four primers were performed with the same PCR program as for the Alu sequence. The PCR products were run on a 1% agarose gel, blotted as described before and hybridized at 55 °C for 2 h with a 5′- and 3′-DIG-labelled 30 bp oligonucleotide probe specific for the CaMV sequence (Eurogentec, Hampshire, UK).

Detection of green fluorescent protein

After 48 h, incubation fluorescing cells growing in tissue culture dishes were observed using an inverted fluorescence microscope excitation at 500 nm.

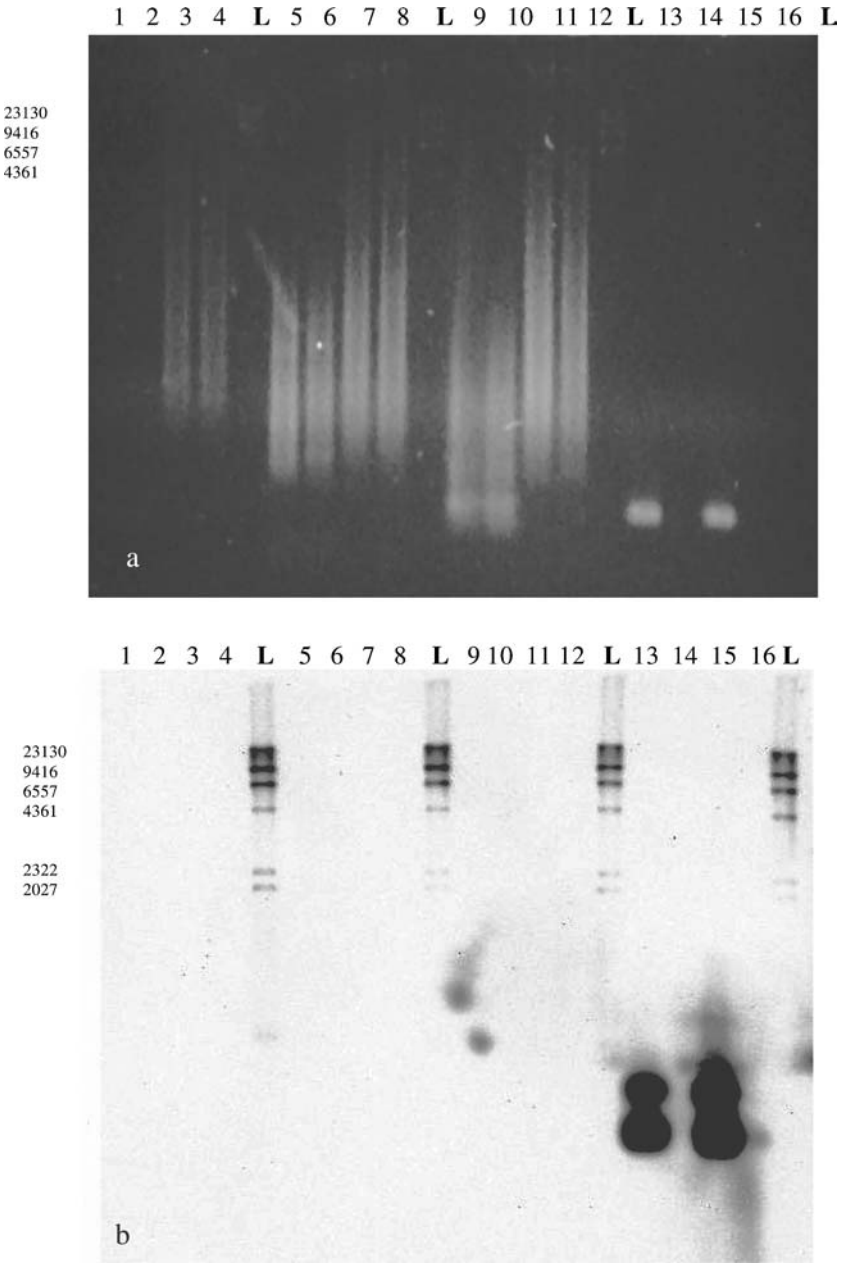

Fig. 4 Southern blot analysis of Alu PCR products amplified from DNA isolated from cells transfected with the various plasmids. An Alu PCR, a 35S GFP PCR, and a combination of the two primer sets were run on DNA isolated from transfected and untransfected cells, and the PCR products were run on a 1% agarose gel and stained with ethidium bromide **(a)**. Lanes 1–4 show PCR products amplified using Alu 1F and 2R primers. Lanes 5–8 show PCR products amplified using the Alu 1F and x-GFP35s-2R primers. Lanes 9–12 show PCR products amplified using the x-35sGFP-2F and Alu R primers. Lanes 13–16 show PCR products amplified using the x-35sGFP-2F and x-GFP35s-2R primers. The agarose gel was blotted and hybridized with a 5′ and 3′ DIG-labelled 30 bp oligonucleotide probe (Eurogentec, Hampshire, UK) specific for the CaMV sequence **(b)**. For both figures: Lane 1,5, 9, and 13, phrGFP-CaMV 100 pg. Lanes 2, 6, 10, and 14; ddH_2O. Lanes 3, 7, 11, and 15; phrGFP-CaMV transfected Caco-2 cells. Lanes 4, 8, 12, and 16; mock-transfected Caco-2 cells

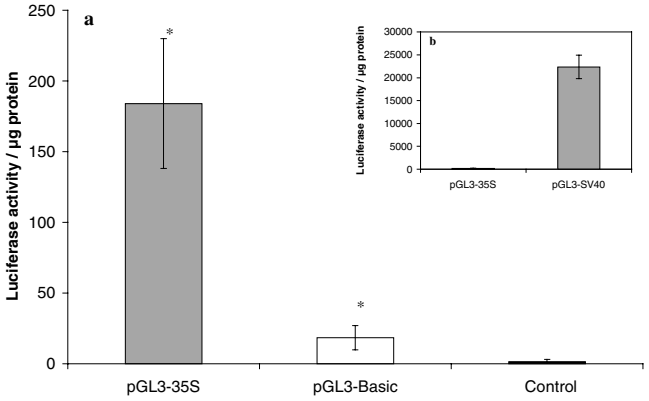

Fig. 5 Spectrophotometric quantification of Luciferase activity in Caco-2 cells. **(a)** Untransfected or transfected with pGL3–35S or the pGL3-basic (promoterless) plasmids. The results represent the mean plus standard deviation of four experiments with minimum three parallels of each reporter plasmids. The measured Luciferase activities were normalized for total protein. The typical protein content was 0.7–2.3 $\mu g\ \mu l^{-1}$. *Statistically significant difference (p=0.0004). **(b)** Comparison of Luciferase activity in Caco-2 cells transfected with pGL3-SV40 and pGL3–35S

Detection and quantification of luciferase activity

The Caco-2 cells were transfected with relevant plasmids using the Lipofectamine Plus reagent as described above. Cells were harvested 48 h after transfection, and the luciferase activities were measured using the Luciferase Assay Kit (Applied Biosystems, Warrington, UK) in a Tropix TR717 Microplate Luminometer (PE, Applied Biosystems, Warrington, UK). Cell cultures were rinsed twice with PBS before adding Lysis solution with 0.5 mM DTT. The cells were incubated on ice for 10 min and the cell lysates were transferred to microfuge tubes and centrifuged for 2 min to pellet the debris. The supernatants were transferred to fresh tubes and used immediately or stored at −70 °C. Proteins were determined according to Bradford [32] by using the Bio-Rad protein assay (Bio-Rad, Hercules, USA). The Luciferase activities measured were normalized for total protein. The typical protein content was between 0.7 and 2.3 $\mu g\ \mu l^{-1}$.

TFSEARCH on the 35S CaMV sequence

A transcription factor binding site software programme was used to identify putative binding motifs for mammalian transcription factors, especially human transcription factor binding sites, in the 564 bp 35S CaMV promoter sequence present in phrGFP-35S and pGL3-35S plasmids (www.cbrc.jp/research/db/TFSEARCH.html).

Results

GFP expression governed by the 35S CaMV promoter

The phrGFP-35S and phrGFP-hCMV plasmids were transfected in parallel into Caco-2 cells. After 48 h of incubation, GFP expression was clearly visualized by fluorescence microscopy. Both phrGFP-hCMV and phrGFP-35S transfected cell cultures were displaying GFP expression (Fig. 2a and c), while GFP expression was not recorded in Caco-2 cells transfected with the promoterless phrGFP plasmid (Fig. 2e). As illustrated by Fig. 2, the number of cells expressing the GFP protein was consistently higher for phrGFP-hCMV transfected than for phrGFP-35S transfected cultures, while the intensity of GFP expression in the fluorescing cells seemed to be at comparable levels for the two plasmids. Southern blots (Fig. 3) indicated that the transfection efficiencies were at the same level for the three plasmids used.

A hypothetical possibility existed that the observed GFP signals were due to chromosomal integration of plasmid fragments, leading to *gfp* gene transcription from an endogenous host cell promoter. This was investigated by Southern blots and Alu PCRs. Total DNA preparations from the cell cultures were run on an agarose gels, blotted and hybridized with probes targeting the different plasmids (Fig. 4). No signals indicating integration were observed. Alu PCRs with total DNA preparations from transfected cells was performed to rule out single genomic inserts. Whereas agarose gel-electrophoresis revealed a weak smear using the Alu primers alone or in combination with CaMV 35S primers, subsequent southern blotting and hybridization with a 35S probe did not reveal genomic inserts (Fig. 4a and b, lane 3, 7, and 11). The smear was probably due to random amplification of Alu sequences in the genome.

Luciferase activity governed by the 35S CaMV promoter

To enable a more quantitative assessment of the 35S CaMV promoter activity in Caco-2 cells, we constructed/obtained

Table 1 Relative Luciferase activity in Caco-2 cells driven by various promoters

Plasmid	Relative activity (%)[a]			
	pGL3-SV40	pGL3-35S	pGL3-Basic	Control[b]
pGL3-SV40	*100*	12913.097±4495.153	143160.193±64654.997	4161137.760±2744291.49
pGL3-35S	0.841±0.257	*100*	1245.902±774.439	32274.382±23542.036
pGL3-	0.082±0.035	10.962±7.111	*100*	4082.265±3488.482
Control[b]	0.006±0.008	0.632±0.713	11.670±18.188	*100*

[a]Each value represents the mean of four repeated experiments±SD

[b]Control: no plasmid added

Table 2 TFSEARCH on 564 bp of the 35S CaMV promoter region

bp	35S CaMV Sequence	Entry	TF	Score
1	GAATTCCCAT GGAGTCAAAG ATTCAAATAG AGGACCTAAC AGAACTCGCC			
	<----------	M00155	ARP-1	85.9
51	GTAAAGACTG GCGAACAGTT CATACAGAGT CTCTTACGAC TCAATGACAA			
101	GAAGAAAATC TTCGTCAACA TGGTGGAGCA CGACACGCTT GTCTACTCCA			
151	AAAATATCAA AGATACAGTC TCAGAAGACC AAAGGGCAAT TGAGACTTTT			
	---------->	M00134	HNF-4	86.7
201	CAACAAAGGG TAATATCCGG AAACCTCCTC GGATTCCATT GCCCAGCTAT			
	<-----	M00076	GATA-2	91.3
	<-------	M00128	GATA-1	86.6
	<---------	M00076	GATA-2	86.6
	<-----	M00076	GATA-2	91.3
251	CTGTCACTTT ATTGTGAAGA TAGTGGAAAA GGAAGGTGGC TCCTACAAAT			
	--------->	M00077	GATA-3	94.4
	---------->	M00076	GATA-2	94.1
	-------------->	M00126	GATA-1	93.7
----		M00076	GATA-2	91.3
-----		M00128	GATA-1	86.6
	-------------->	M00127	GATA-1	85.5
301	GCCATCATTG CGATAAAGGA AAGGCCATCG TTGAAGATGC CTCTGCCGAC			
	<----------	M00053	c-Rel	89.3
	--------->	M00077	GATA-3	85.9
	---------->	M00076	GATA-2	85.8
351	AGTGGTCCCA AAGATGGACC CCCACCCACG AGGAGCATCG TGGAAAAAGA			
-----	>	M00271	AML-1a	85.4
401	AGACGTTCCA ACCACGTCTT CAAAGCAAGT GGATTGATGT GATATCTCCA			
	---------->	M00106	CDP CR	91.3
	<----------	M00124	Pbx1b	89.1
	<----------	M00076	GATA-2	88.1
	<---------	M00217	USF	87.9
	---------->	M00076	GATA-2	87.7
	<---------	M00096	Pbx-1	86.3
451	CTGACGTAAG GGATGACGCA CAATCCCACT ATCCTTCGCA AGACCCTTCC			
-------	>	M00039	CREB	98.0
-------	>	M00041	CRE-BP	93.6
	<----------	M00076	GATA-2	91.3
-------	>	M00040	CRE-BP	91.2
	-------------->	M00113	CREB	91.0
-------	<	M00040	CRE-BP	91.0
	<---------	M00077	GATA-3	89.1
	<----------	M00147	HSF2	87.2
	<----------	M00076	GATA-2	87.0
-------	<	M00041	CRE-BP	86.9
-------	<	M00039	CREB	86.6
	<---------	M00077	GATA-3	85.6
	----------------->	M00114	Tax/CR	85.3
501	TCTATATAAG GAAGTTCATT TCATTTGGAG AGGACAGGGT ACCCGGGGAT			
---------	>	M00216	TATA	88.8
	------	M00051	NF-kap	87.0
	<-----	M00051	NF-kap	87.0
	---------------->	M00252	TATA	86.6
	-------------->	M00159	C/EBP	85.4
551	CCTCTAGAGT CGAC			
---	>	M00051	NF-kap	87.0
----		M00051	NF-kap	87.0

[a]ver.1.3 (c) 1995 Yutaka Akiyama (Kyoto University). A total of 61 high-scoring sites were found. Max score: 98.0 point, Min score: 85.3 point. This simple routine searches highly correlated sequence fragments versus the TFMATRIX transcription factor binding site profile database by E. Wingender, R. Knueppel, P. Dietze, H. Karas (GBF-Braunschweig)

a new set of plasmids with the 35S CaMV promoter or the SV40 promoter in front of the *Luciferase* gene. An isogenic promoterless plasmid was used as a negative control.

The results of the spectrophotometric assay of Luciferase production in the Caco-2 cells are presented in Fig. 5. A significant difference (p=0.0004) in the Luciferase activity was found between the promoterless plasmid pGL3-Basic, and the 35S CaMV promoter containing plasmid pGL3-35S (Fig. 5a). When compared to the SV40 promoter (Fig. 5b), the 35S CaMV promoter showed 0.84% of the SV40 activity (Table 1).

TFSEARCH on the 564 bp 35S CaMV promoter sequence present in phrGFP-35S and pGL3-35S plasmids

The transcription factor binding site software programme TFSEARCH demonstrated the presence of more than 40 putative mammalian transcription factor-binding sites (data not shown), and indicated that 18 different human transcription factors may bind to sequence motifs found in the 564 bp 35S promoter sequence (Table 2).

Discussion

The purpose of the present investigation was to determine whether the 35S CaMV promoter possesses the potential to drive gene expression in cultures of human intestinal, enterocyte-like cells. It was demonstrated that the 35S CaMV promoter was able to drive expression of the *gfp* as well as the *luc* reporter genes in the enterocyte-like human cell line Caco-2.

The expression levels from the 35S promoter were substantially lower than those obtained using the strong viral promoters of SV40 and hCMV. Direct efficiency comparisons of promoters normally functioning in totally different biological contexts do not make much sense, however. The significant observation made was that the 35S CaMV promoter, generally assumed to be plant specific (e.g. [5]), initiates significant protein expression levels in host cells that share important characteristics with those lining parts of the human GIT. Our results corroborate and extend the recent results of Vlasak et al. [13] and Tepfer et al. [14]. Taking the published studies together, it may now be concluded that the 35S CaMV promoter is capable of initiating gene expression in some mammalian cell lines under a range of different conditions and circumstances. The different reporter gene systems, plasmids, and experimental designs as well as cell species and lines employed, may provide explanations for the somewhat deviating results obtained in the three studies.

Selecting mammalian cell cultures of potential relevance and authenticity is of uttermost importance in this type of biosafety-related studies. But, irrespective of how careful and considerate selection of cell cultures has been performed, the paramount difference between in vivo and in vitro situations cannot be over-emphasized. Hence, well-designed cell culture experiments may give lead for, but never replace, in vivo studies.

Furthermore, whether the demonstrated potential of the 35S CaMV fragment to promote gene expression in human enterocyte-like cells may have GE food safety implications, is directly related to the process of foreign DNA uptake from the human gastro-intestinal tract. Recent experiments in some animal species demonstrated that uptake of foreign DNA from the GIT may be considered a physiological process. Although the mechanisms remain to be elucidated, a number of studies in laboratory rodent and domestic animal species show that considerably-sized fragments of food-derived DNA may be taken up from the mammalian GIT and transported by blood to internal organs. Very recently, this phenomenon was also observed in human volunteers [22]. But so far, uptake of fragments containing the intact 35S promoter has not been directly demonstrated in any species.

From a biosafety point of view, whether related to human health or ecological considerations, it is generally desirable to put transgene expression in GE crops under the control of strictly species- and tissue-specific promoters. However, the basis for apparent species and tissue specificity of various plant or plant virus promoters used in transgenic organisms remains to be fully understood, and even if that was not the case, the prospect of totally "non-leaky" promoters may be illusory. Furthermore, promoter specificity in plants does not exclude gene expression activity in organisms consuming GE plants. Whether any given DNA sequence may act as a transcription regulatory element, i.e. promoter or enhancer, in a new intracellular environment, is to a high extent determined by the combination of sequence motifs present in the DNA and the menu of transcription factors expressed in the particular cell under the given environmental conditions. Consequently, sequence analyses for transcription factor-binding motifs may be extremely useful for biosafety forecasts of candidate promoters to be used in transgenic plants.

The transcription factor-binding site software programme TFSEARCH revealed that the 35S CaMV promoter contains a mosaic of overlapping putative binding motifs for mammalian transcription factors. Among these putative sequence motifs were 18 for binding of different human transcription factors. These 18 factors include generally important ones, e.g. CREB, but also a number of lymphocyte-specific factors, e.g. GATA members. Taken together, putative interactions of the promoter with such a considerable number of differentially expressed transcription factors may imply complex and variable transcriptional activity of the 35S promoter in various mammalian species, tissues, and cells. Such putative interactions, and their consequences for host cells and organisms, should be clarified by carefully designed in vitro and in vivo experiments.

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