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Transient expression in mammalian cells of transgenes transcribed from the *Cauliflower mosaic virus* 35S promoter

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Gene constructs containing the *Cauliflower mosaic virus* (CaMV) 35S promoter and a sequence coding either for a green fluorescent protein (GFP) or for firefly luciferase were transfected into Chinese hamster ovary (CHO) cells. Both reporter genes were expressed to significant levels. The 35S promoter was 40 times less active than the human eF1α promoter, which is known to be one of the most potent promoters in mammalian cells. The 35S promoter must therefore be considered to be a promoter of significant potency in mammalian cells. RT-PCR analysis suggested that transcription initiation in CHO cells occurred between the TATA box and the transcription start site of the 35S promoter that function in plant cells. Further analysis by 5'RACE confirmed that transcription was initiated in CHO cells at different sites located essentially between the TATA box and the plant transcription start site, showing that 35S promoter activity in animal cells is due to the presence of promoter elements that are functional in mammalian cells, but that are not those used in plants. The data reported here raise the possibility that genes controlled by the 35S promoter, which is commonly used in transgenic plants, have the potential for expression in animal cells.

Keywords: CaMV / 35S promoter / mammalian cells / transcription

Abbreviations: CaMV: *Cauliflower mosaic virus*; **EGFP:** enhanced green fluorescent protein; **CHO:** Chinese hamster ovary; **nos:** nopaline synthase; **SV40:** simian virus 40; **5'RACE:** 5' rapid amplification of cDNA ends; **DRE:** downstream regulatory element; **RT-PCR:** reverse transcription-polymerase chain reaction

INTRODUCTION

Cauliflower mosaic virus (CaMV), which infects numerous cruciferous plants, has a circular DNA genome of approximately 8 kb (Hull, 2002). Early in the 1980s, CaMV attracted considerable attention as a potential vector for expressing foreign genes in plants, but it proved to be not very useful, since the CaMV genome can accept only very limited additional sequences, and the viral RNAs are translated in a highly complex fashion (Hull, 2002). Nonetheless, these early studies showed that the CaMV genome is transcribed from two strong promoters, the 19S and 35S promoters (Guilley et al., 1982), which have been widely used to drive gene expression in transgenic plants. Indeed the 35S promoter has become the most widely used promoter in transgenic plants; it is present in the majority of those developed so far (see http://www.isb.vt.edu/). In part because of its importance, the essential regulatory elements of the 35S promoter, which are concentrated in the 343 nucleotides preceding the transcription initiation site, were studied intensively during the 1980s (Benfey et al., 1989; Fang et al., 1989; Odell et al., 1985; Prat et al., 1989).

Although many core elements regulating transcription are conserved among eukaryotes, the mechanisms that control gene transcription are generally considered to be specific to the different phyla of living organisms, since many animal genes are not functional in plants and *vice versa.* For example, the promoters of the following animal genes were not functional in plants: alpha-actin (Koncz et al., 1984), mouse metallothionein, simian virus (SV40) early, and Herpes simplex thymidine kinase (An, 1986).

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Yet it is known that some promoters are active in cells of quite distant origin. The promoter of the animal Rous sarcoma virus is active in *E. coli*, the promoter of a *Drosophila* heatshock protein gene (*hsp70*) is active in plant cells (Spena et al., 1985), as is a human cytomegalovirus (hCMV) promoter (Vlasák et al., 2003), and certain plant gene promoters have been shown to function in animal cells. For instance, a maize zein promoter was transcriptionally active *in vitro* in an extract of human HeLa cells (Langridge and Feix, 1983). This is also the case for the maize alcohol dehydrogenase 1 (*Adh1*) gene promoter, which could be used to express resistance genes in monkey cells (Dennis and Berg, 1985). Furthermore, the activity of the Adh1 promoter was strongly stimulated by the SV40 enhancers. Similarly, the promoter of the Ti plasmid *nos* gene, which is transferred from *Agrobacterium tumefaciens* to plant cells where it is expressed, is also active in *Xenopus* oocytes (Ballas et al., 1989), and in HeLa cells (Zahm et al., 1989).

The CaMV 35S promoter is functional in a remarkably broad range of organisms. The evidence indicates that the 35S promoter is functional in *E. coli*, using several transcription initiation sites similar to those found in prokaryotic promoters (Assaad and Signer, 1990). The 35S promoter has also been shown to be active in two yeasts: *Saccharomyces cerevisiae* (Rüth et al., 1992; 1994) and *Schizosaccharomyces pombe* (Pobjecky et al., 1990), in which initiation occurs at the same site as in plants (Hirt et al., 1990). Homology between yeast and plant transcription machinery may explain this phenomenon (Hirt et al., 1990; Rüth et al., 1994). More recently, the 35S promoter has also been shown to function in two basidiomycete fungal species, *Ganoderma lucidum* and *Pleurotus citrinopileatus* (Sun et al., 2002).

There is also considerable evidence, much of it indirect, suggesting that the 35S promoter could be functional in vertebrate cells. A reporter gene driven by the 35S promoter was as potent as the SV40 early gene promoter in *Xenopus* oocytes (Ballas et al., 1989). Moreover, the 35S promoter forms active preinitiation complexes in a human *in vitro* transcription system (Katagiri et al., 1990), and is transcribed *in vitro* in HeLa cell-free extracts, using the same transcription initiation site as in plants (Burke et al., 1990; Cooke and Penon, 1990; Guilley et al., 1982). It is interesting to note that the plant transcription factor TGA1 increases the number of preinitiation complexes in a human *in vitro* transcription system (Katigiri et al., 1990), and also enhances reporter gene expression in yeast (Rüth et al., 1994). In addition, the transcription factor IIA of wheat could replace human TFIIA for transcription in HeLa cell-free extracts (Burke et al., 1990).

These results raise the question of whether foreign genes driven by the 35S promoter would be expressed in intact mammalian cells. Quite recently, Vlasák et al. (2003) presented results showing very low levels of expression when they introduced several 35S constructs into mammalian cells. In contrast, the results presented here show relatively high levels of expression of genes transcribed from the 35S promoter in Chinese hamster ovary (CHO) cells. This observation motivated investigation of the sites of transcription used, since if they were far upstream from those used in plants, as is the case for the major site used in *E. coli* (Assaad and Signer, 1990), this would open the possibility of designing a 35S promoter that would function in plants but not in animal cells.

RESULTS

Cell transfection

CHO cells were transfected with three plasmids containing the EGFP (enhanced GFP) coding sequence, one without a promoter, one with the 35S promoter, and one with the eF1α promoter. Gene expression was evaluated two days after transfection. Both EFIα-EGFP and 35S-EGFP genes were expressed in a number of cells (Fig. 1A and B), whereas the plasmid without any promoter gave no signal (Fig. 1C). The intensity of fluorescence of the transfected cells suggested that the eF1α-EGFP vector was more potent than the 35S-EGFP vector. These results show that a 35S-EGFP gene inserted into a typical vector for plant transformation, pCambia-1300 (accession number AF234296), is expressed when transfected into animal cells.

In order to compare more precisely the potency of the two promoters, the firefly luciferase gene was used as a reporter in similar transient expression experiments. The eF1α-luciferase plasmid was 40 times more potent than the 35S-luciferase plasmid (Fig. 2). In our hands, the eF1α-luciferase construct was twice as active as the pcDNA3-luciferase construct, more frequently used by experimenters, in which the transgene is driven by the hCMV promoter (Houdebine, unpublished data). This means that the 35S promoter acted as a promoter of medium potency in CHO cells.

Localization of the transcription initiation site by RT-PCR

Inspection of the sequence of the 35S promoter showed that there are sequence motifs that could play a role in

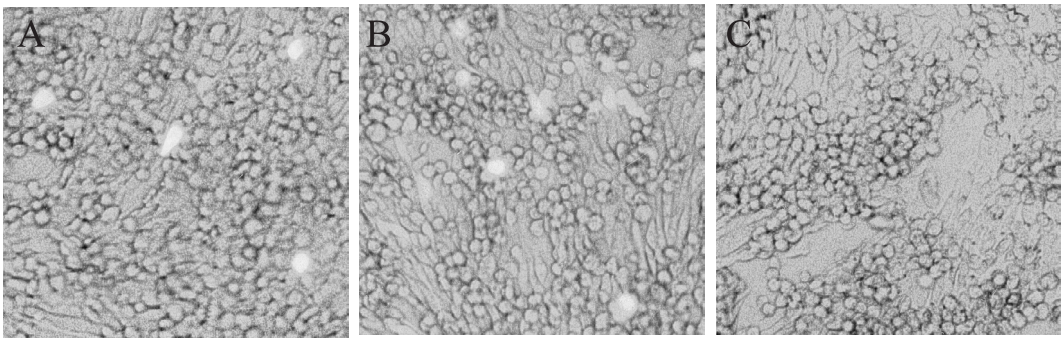

Figure 1. EGFP gene expression in CHO cells transfected by the plasmids 35S-EGFP, EF1α-EGFP and EGFP. The fluorescence was observed two days after transfection with A: EF1α-EGFP; B: 35S-EGFP; and C: promoterless EGFP. GFP-expressing cells appear light on the grey background of non-expressing cells.

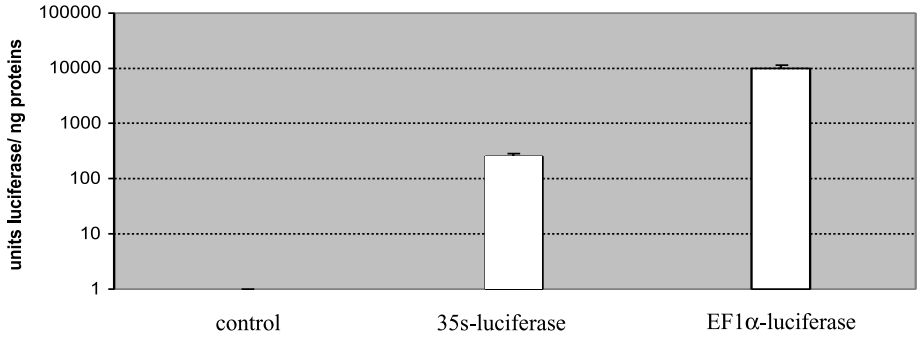

Figure 2. Firefly luciferase gene expression. CHO cells were transfected with: (1) Control plasmid without luciferase gene, (2) 35S-luciferase, and (3) EF1α-luciferase. Results are the means +/– SEM of three independent transfections and duplicates.

transcription in animal cells (for review see Butler and Kadonaga, 2002). In addition to the plant TATA box, there are two putative Inr sites upstream of the 35S transcription start site used in plants (Fig. 3). Both have the TCATTT consensus sequence. DRE motifs are often found in association with Inr sites, but none was found in the 35S 5' untranslated region. However, the plant TATA box is 15 and 20 bp upstream of the two putative Inr sequences, and is thus positioned appropriately to act as an enhancer for the two Inr sites.

In order to determine if the plant 35S transcription start site was used in CHO cells, RT-PCR was performed using primer BTG1 within the expected mRNA, and several primers positioned at or near possible sites for the 5' end of the mRNA. The sequence shown in Figure 3 corresponds to the sequence of the construct transfected into CHO cells. In conjunction with primer BTG1, the primers TKAL1 and 35S generated RT-PCR amplification products, whereas primer TATA-35S did not (Fig. 4). This indicates that the initiation site in CHO cells was essentially upstream of the plant 35S transcription start site and downstream of the TATA box.

Identification of the transcription initiation site using 5'RACE

The RT-PCR results described above suggested that at least one of the Inr sites located between the TATA box and the plant transcription start site might be used for transcription from the 35S promoter in CHO cells. In order to clarify this point, we used 5'RACE on the same RNA samples to identify more precisely the 5' end of the mRNA in CHO cells. First, cDNA was synthesized by reverse transcription primed with oligo-dT. Then, PCR was carried out using the primer GLP2 shown in Figure 3, and the primer UPM provided in the SMART 5'RACE kit

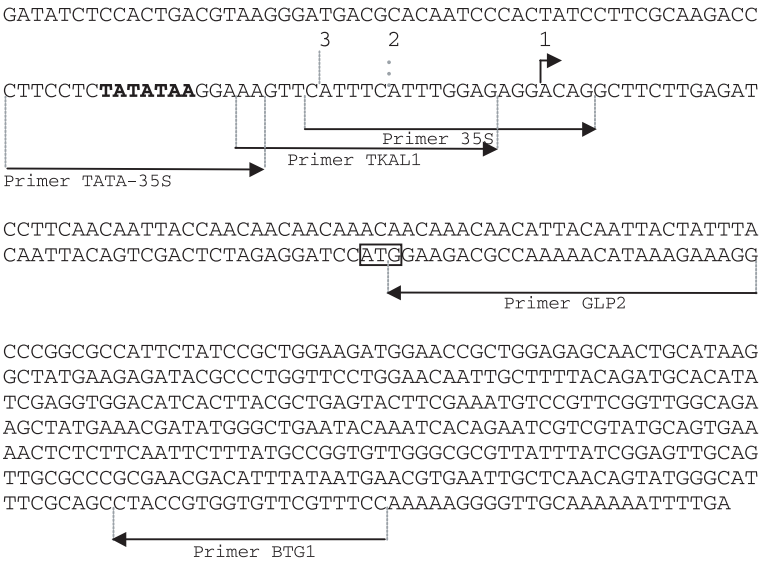

Figure 3. Primary structure of the 35S-luciferase gene and primers used in RT-PCR and 5'RACE experiments. The short bent arrow indicates the 35S transcription start site, the plant TATA box is in bold, and the translation initiation codon is boxed. Numbers 2 and 3 designate putative Inr transcription initiation sites. The long horizontal arrows designate the primers used for RT-PCR and 5'RACE.

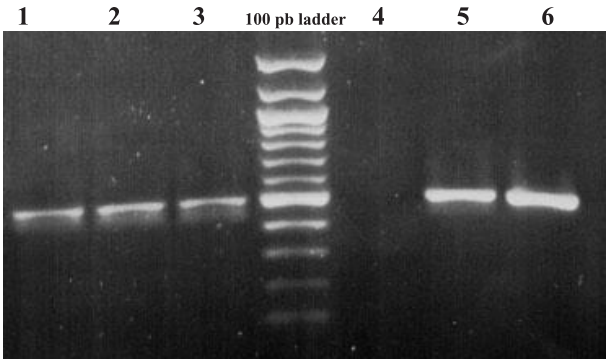

Figure 4. Agarose gel electrophoresis of the RT-PCR products obtained from total RNA extracted from CHO cells transfected with the 35S-luciferase construct. Lanes 1, 2 and 3: PCR product obtained from the plasmid. Lanes 4, 5, and 6: RT-PCR product obtained from total RNA extracted from transfected CHO cells. The central lane contains size marker DNA. The different primers used are the following: lanes 1 and 4: TATA-35S and BTG1; lanes 2 and 5: TKAL1 and BTG1; lanes 3 and 6: 35S and BTG1.

(Clontech). The band corresponding to the amplified material was cloned and sequenced. The sequence of nine clones is shown in Figure 5.

Unexpectedly, six different 5' ends were found among the clones sequenced. None corresponded to the major transcription start site used in *E. coli,* which is at position –315, nor to the minor site at –17 (Assaad and Signer, 1990). In addition, none of the clones could correspond to transcription initiation at either of the putative Inr sites. Instead, initiation occurred at different sites, of which all but one were between the TATA box and the 35S transcription start site in plants. It should be noted that the results are not due to the presence of contaminating DNA, since there was no amplification by RT-PCR using primers TATA-35S and BTG1 (Fig. 4, lane 4). It is not known if this proportion is representative of the entire population of initiation sites. However, multiple initiation sites have been observed for a number of genes (Butler and Kadonaga, 2002), and it appears that multiple initiation complexes are formed in a cell-specific manner (Hochheimer and Tjian, 2003).

DISCUSSION

The experiments described in the present paper show without ambiguity that the CaMV 35S promoter functions as a promoter of medium potency in mammalian cells. This is in contrast with the results of Vlasák et al. (2003), who found only very low levels of transcription from 35S constructs transfected into human embryonal kidney cells. There are several differences between their experimental methods and those used here, including the use of different cells, different vector plasmids, and different coding sequences. However, none provides an obvious explanation for the difference between their results and those described here.

The 35S promoter could well be a universally functional promoter, since it is active, not only in higher plants, but also in bacteria, ascomycetes, basidiomycetes, as well as in amphibian and mammalian cells. The ability of the

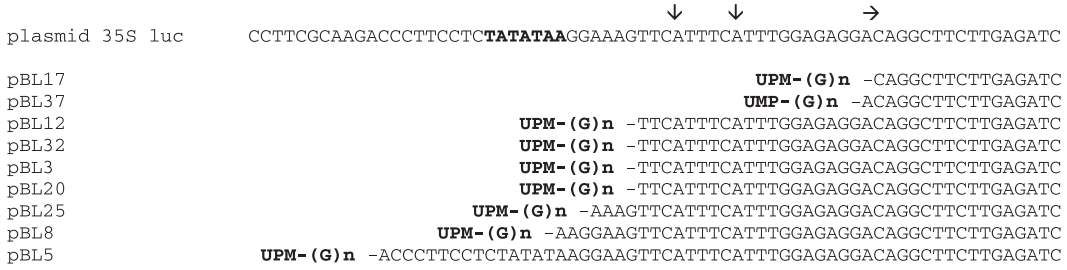

Figure 5. Sequence of the products obtained by 5'RACE. The first line indicates the sequence of the 35S promoter region. The plant TATA box is in bold, straight vertical arrows indicate putative Inr sites, and the plant transcription start site is indicated by a horizontal arrow. The nine following lines show the 5' sequence of the nine inserts cloned from the 5'RACE product. UPM-(G)n designates the 5' primer used for 5'RACE.

35S promoter to function in this broad range of organisms could perhaps be attributed to the remarkable conservation throughout eukaryote evolution of the core transcriptional regulatory elements (for reviews see Martinez, 2002; Butler and Kadonaga, 2002). However, in this regard it is interesting that the transcription start site observed in CHO cells was neither the TATA-box-dependent site used in plants, nor one determined by one of the putative Inr motifs. An additional puzzling point is that regulation of 35S promoter expression is not constant across these different groups of organisms, since for instance it is consistently expressed to high levels in plants, but is cAMP-dependent in *S. cereviseae* (Rüth et al., 1992).

Ho et al. (1999; 2000) described a scenario composed of a lengthy chain of events, implying potential risks associated with the 35S promoter. They claimed that the 35S promoter is a hotspot for DNA recombination, that it would be unstable in the genome of transgenic plants, that when humans consume plants containing the 35S promoter this would lead to genetic transformation of human cells, and that this sequence of events would lead to various consequences. These claims have been refuted in detail elsewhere (Hull et al., 2000; Morel and Tepfer, 2000). Although the results described here do confirm that the 35S promoter is expressed in mammalian cells, they do not in any way validate Ho et al.'s scenario, since the numerous other steps required for harm to occur by this mechanism are not supported by the scientific evidence.

However, the possibility that genes controlled by the 35S promoter could be expressed in animal cells raises a different biosafety question that we will consider briefly. There is one report that *Agrobacterium tumefaciens* can transform several mammalian cell types *in vitro* (Kunik et al., 2001). If confirmed, this result raises the question of *Agrobacterium* transformation of cells in intact animals. However, at this time there is no direct evidence that this could occur. Nonetheless, we suggest that *Agrobacterium* strains should be handled with particular care when they harbor genes that could be expressed in mammalian cells or could have a direct effect on humans (*e.g.*, genes encoding pharmaceutical proteins). However, once progeny of stably-transformed plants have been obtained, *Agrobacteria* are normally no longer present, and thus the potential risk evoked here is only relevant to laboratory and greenhouse activities. In this regard, it is important to note that in more than 20 years of research using *Agrobacterium* in numerous labs, there has never been any report of deleterious health effects.

MATERIALS AND METHODS

Plasmid vectors

The p35S-EGFP vector was prepared by inserting the *Hin*dIII-*Nco*I fragment from pCaMV35S-sGFP(S65T)-nos3' (Chiu et al., 1996) contained the 35S promoter, the EGFP (enhanced green fluorescent protein) coding region and the *Nos* gene 3' noncoding region into pCambia-1300 (accession number AF234296). The same construct without a promoter was used as control (p-EGFP).

The p35S-luciferase vector was prepared by inserting the 500bp *Hin*dIII-*Nco*I fragment containing the 35S promoter into the *Hin*dIII-*Nco*I sites of the pGL3 basic plasmid (Clontech).

The EGFP and luciferase coding sequences were also introduced into a vector containing the human EF1α gene promoter and first intron, the human growth hormone 3' noncoding region and two copies of the 5'HS4 insulator from the locus control region of the chicken β-globin

locus. This vector, p382m, was derived from piEF0 (Taboit-Dameron et al., 1999), by deleting the CT-rich region following the cap site, in order to suppress translation inhibition in quiescent cells.

CHO cell culture and transfection

CHO cells were seeded at half confluency and transfected the next day using Lipofectamine as transfecting agent according to the recommendations of the manufacturer (Life Technologies). Two days later, cells transfected with the EGFP gene were observed with an inverted microscope under U.V. illumination using an FITC filter. Cells transfected with the luciferase gene were collected and the enzyme activity was measured in extracts as previously described (Pantano et al., 2002). Results are the means ± SEM of three independent transfections and duplicates.

Localization of the transcription initiation site by RT-PCR

Total RNA was extracted from CHO cells transfected by the 35S-luciferase plasmid two days after transfection. Different regions of the luciferase mRNA were amplified by RT-PCR using the primers indicated in Figure 3. The amplification products were visualized after agarose gel electrophoresis.

Identification of the transcription initiation site using 5'RACE

The 5'RACE kit from Clontech (SMART 5'RACE) was used. The 5' primer provided by the manufacturer (Universal Primer Mix: UPM) and the 3' primer GLP2 shown in Figure 3 were used for reverse transcription and amplification. The amplified material was cloned in pGEMT and sequenced.

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