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Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter

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Abstract

As a highly active plant viral promoter that is able to function in a wide variety of cell types, the cauliflower mosaic virus (CaMV) 35S promoter has the potential for harboring a plant enhancer element. We tested this possibility and demonstrated that a 338 base pair fragment isolated from the region upstream of the 35S TATA box can increase the expression of a low-activity heterologous promoter up to the level observed for the intact 35S promoter. This fragment is fully active in both orientations when placed 150 base pairs upstream of the transcription start site. However, the activity of this fragment is sensitive to location, demonstrating a reduction in activity and loss of orientation-independent function when the distance from the transcription start site is increased. By assaying fragments of different sizes, we have also characterized regions that are functional in directing the stimulation of the heterologous promoter.

Introduction

The promoter that drives the expression of the 35S RNA of cauliflower mosaic virus (CaMV) has been isolated and used to express heterologous RNAs in cells of both dicots and monocots including tobacco, petunia, soybean, carrot, maize, and rice [1, 5, 14, 18, 20]. It is highly active in both transient expression systems and stably transformed plant cells, showing up to a 30-fold higher level of expression than the nopaline synthase promoter [15, 22]. The nopaline synthase (NOS) promoter is derived from the *Agrobacterium tumefaciens* Ti-plasmid, and is commonly used to express the bacterial neomycin phosphotransferase II coding region to confer kanamycin resistance for the selection of transformed plant cells. The only other promoters found to have activity as high as that of the 35S promoter in transformed plants are those from genes for the chlorophyll a/b binding protein and the small subunit of ribulose bisphosphate carboxylase (rbcS), which are both tissue-specific and highly regulated by light [3, 10].

DNA elements that are responsible for the high light induction of two rbcS genes in leaves have been identified [3, 25]. These have been called enhancer-like elements due to their abilities to confer high-level expression on low-activity promoter fragments when placed in either orientation in a proximal 5′ location (−145 or −46). One element was tested in a 3′ location and found to be ineffective [25], indicating that it does not have all of the properties of previously described enhancers. The prototype enhancer, having orientation-independent activity at a distance greater than 1 kilobase (kb) 5′ or 3′ to the transcription start site, was first identified in highly expressed animal virus promoter regions such as in the SV40 early promoter region [12], suggesting that the CaMV 35S promoter region might harbor a simi-

lar type of element.

Previous 5′ deletion analysis of the 35S promoter, carried out in stably transformed tobacco calli and plants, identified the region with boundaries of −343 and −46 as containing sequences required for high expression [18]. In this report we show that this sequence region is able to stimulate expression of the low-activity NOS promoter, and we further test it for properties ascribed to a true enhancer [7]. Within the −105 to −46 region of this 35S promoter sequence are some interesting features including a CAT box, an inverted repeat, and a sequence with homology to the SV40 core enhancer [18]. In this report we analyze further the functions of these sequence features by deletion and by placing isolated fragments in conjunction with the NOS promoter. Constructions were made which separate the CAT box from the SV40 core enhancer homology sequence and interrupt the inverted repeat. To eliminate the problem of variation among independent transformants bearing the same gene construction that is encountered with stable transformants [10, 16, 19], we used a protoplast transient expression assay system. Stable transformants were also generated and assayed to extend some of our results to the whole plant level.

Materials and methods

Plasmid constructions

A pBR322-based plasmid containing a 296 base pair (bp) Sau 3A-Pst I nopaline synthase (NOS) promoter fragment, followed by a unique Hind III restriction site and then a 702 bp BamH I-Cla I NOS polyadenylation signal sequence region fragment, was kindly provided by N. Yadav. A chloramphenicol acetyltransferase (CAT) coding region was added by first cloning a 974 bp Sau 3A fragment (blunted) from pBR325 into the blunted Sal I site of pGEM2 in the orientation such that the polylinker Hind III and BamH I sites are 5′ and 3′ to the coding region, respectively. Then the Hind III-BamH I CAT fragment was placed adjacent to the NOS promoter resulting in a NOS-P-CAT-NOS 3′ chimeric gene (Fig. 1A). A 35S-P-CAT-NOS 3′ chimeric gene was constructed by replacing the NOS promoter with a 960 bp EcoR I-Hind III fragment containing 35S promoter sequences that has been described [14]. A 338 bp Acc I-Hga I fragment containing sequences between −392 and −55 of the 35S promoter was gel-purified, blunted, and cloned into several sites (after blunting) surrounding the NOS-P-CAT-NOS 3′ gene. These sites as shown in Fig. 1A, with each distance from the NOS transcription start site, are: 1) Sst II at −150, 2) BamH I at +1000, and 3) Sal I

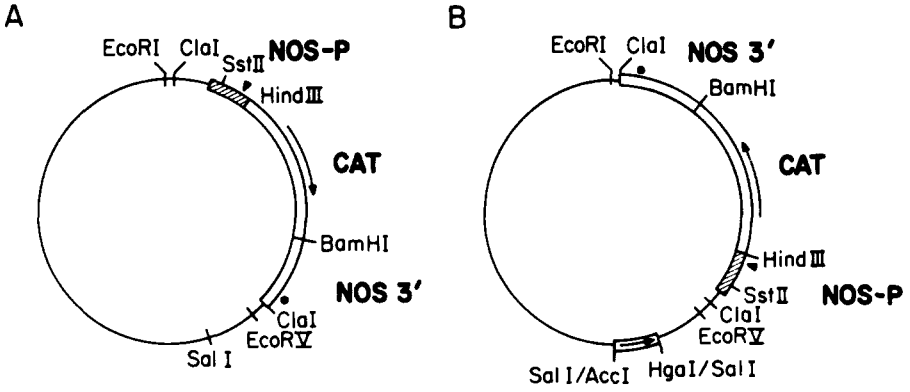

Fig. 1. (A) Diagram of the plasmid containing the NOS-P-CAT-NOS 3′ chimeric gene construction. The triangle marks the NOS-P transcription start site. The arrow indicates the coding orientation of the CAT fragment. The dot marks the polyadenylation site. Relevant restriction sites mentioned in the Materials and methods are labeled. (B) Diagram of the same construction with the ClaI fragment containing the NOS-P-CAT-NOS 3′ chimeric gene inverted, and the 338 bp AccI-HgaI fragment from the 35S promoter region inserted in the SalI site in the forward orientation.

at +2300. Single, and in some cases double, inserts were obtained and the orientation of each insert determined by restriction digests, making use of the EcoR V site located at −90 in the insert.

In plasmids with inserts at Sst II, Sal I, or with no insert, the Cla I fragment containing the NOS-P-CAT-NOS 3′ gene was inverted, an example of which is shown in Fig. 1B. This inversion places the Sal I insert now at −1200. The inversion of the Cla I fragment in the plasmid with the Sst II site insert acts as a control for possible effects of adjacent plasmid sequences on the NOS promoter expression. Inversion of the Cla I fragment in the clone with no insert again acts as a control and also now places the 5′ Cla I site at −580 and the EcoR V site at −740. The Acc I-Hga I fragment was added to these sites and the number and orientation of each insert was determined. A summary of the resulting clones is shown in Fig. 3A.

Other fragments were cloned by blunt-end ligation into the blunted Sst II site at −150. These include 1) a 303 bp Acc I-EcoR V fragment with sequence between −392 and −90 of the 35S promoter, 2) a 76 bp Xmn I-Hga I fragment with sequence between −130 and −55 of the 35S promoter, and 3) a 182 bp Pvu II-Xho I fragment with sequence between −270 and −89 of the SV40 early promoter. Fragment 1) was oriented by the Xmn I site at −130, fragment 2) by the EcoR V site at −90, and fragment 3) by the Sph I sites at −128 and −200. A diagram of these fragments is shown in Fig. 4A. Also shown in Fig. 4A is a 5′ deletion of of the 35S promoter retaining sequences to −90, that was constructed by dropping out the EcoR I-EcoR V region.

Transient expression assay

Soybean protoplasts were prepared from *Glycine max* L. Merr. cv. Wye or Williams 82 cotyledons and DNA was introduced by electroporation as previously described [14]. Tobacco protoplasts were prepared from *Nicotiana tabacum* cv. Xanthi by the method of Nagy and Maliga [17], as modified by Potrykus and Shillito [21]. DNA was directly introduced into tobacco protoplasts in the following manner, which is based on methods of Krens *et al.* [13] and Shillito *et al.* [24]. After a 5 min protoplast heat shock at 45 °C, DNA was introduced by mixing 1−3 million protoplasts per sample with 10 μg/ml plasmid DNA, 50 μg/ml sheared calf thymus DNA, and 13% PEG (PEG 8000, Sigma) for 10 min. The PEG was then diluted 10-fold by addition of H medium [21] and protoplasts were incubated in the dark at 25 °C for three days.

Protoplasts were collected by centrifugation and lysed in 0.2 ml of CAT assay buffer (50 mM sucrose, 0.1 M Tris pH 8, 1 mM DTT, and 0.1% ascorbic acid) by sonication. Protein concentrations were determined using the Bio-Rad reagent and an equal amount of protein from each sample (varying between 8 and 16 μg for separate experiments) was assayed for CAT activity according to Gorman *et al.* [6] as modified by Herrera-Estrella *et al.* [9], except that the reaction contained 0.1 mM acetyl CoA and 1 μCi ^{14}C-chloramphenicol and was incubated for 1 h. Each gene construction was assayed at least 3 times and duplicates of treated protoplasts were carried through each assay. In all figures each set of consecutively numbered samples represents one experiment carried out on aliquots of one protoplast preparation. TLC plates on which acetylated ^{14}C-chloramphenicol reaction products were separated from the ^{14}C-chloramphenicol substrate were scanned for radioactivity counts using an AMBIS Beta Scanning System (Automated Microbiology Systems, Inc.). Total reaction products were calculated as a percent of the total input counts to compare activities of different constructions.

Stable transformation

Tobacco protoplasts were prepared and treated with plasmid DNA as described above. Following incubation in the dark for 3 days and in the light for 4 days at 25 °C, liquid cultures of protoplasts were embedded in agarose (BRL Ultra Pure) and cultured as described by Shillito *et al.* [23, 24], using 50 mg/l kanamycin for selection of transformants. After 5 weeks kanamycin-resistant calli were placed either on a callus maintenance medium containing MS salts, 30% sucrose, 1 mg/L NAA, 0.2 mg/L BAP, and

0.8% agar or on a shoot induction medium containing MS salts, 10 g sucrose, 1 mg/L BAP, and 0.8% agar. Differentiated shoots were placed on rooting medium containing MS salts, 10 g sucrose, and 0.8% agar, and following rooting were transferred to soil. All media contained 100 mg/l kanamycin.

For CAT assays on calli, 40 independent transformed calli containing the same gene construction were pooled. Following grinding at a ratio of 1 g callus per 0.2 ml CAT assay buffer, the protein concentration was determined and CAT activity assayed as described above. CAT activity was assayed similarly in different tissues of individual transgenic plants. From plants that were 25 – 30 cm tall, counting from the top of the plant, leaf 2, 4, 6, and 8, an upper stem segment, a lower stem segment, and a root sample were ground in CAT assay buffer and assayed as described above.

Results

Test for a transcription-stimulating fragment

To determine whether the 35S promoter contains a sequence that can act to increase transcription from a heterologous promoter, the effect of an isolated 5′ fragment on the expression of chloramphenicol acetyltransferase (CAT, EC 2.3.1.28) activity from a NOS-P-CAT-NOS 3′ (NOS promoter, CAT coding region, and NOS polyadenylation signal region) chimeric gene was assessed. A 338 bp Acc I-Hga I fragment that extends from −392 to −55 with respect to the 35S RNA transcription start site, extending to the boundaries of DNA sequence previously shown to include information important for 35S promoter activity by a 5′ deletion analysis [18], was chosen. This fragment was placed in the Sst II site located at −150 of the nopaline synthase promoter, as described in Materials and methods (see Fig. 1A). The gene construction with the chimeric promoter, the control with no insert, and one with the NOS promoter replaced by the entire 35S promoter (35S-P) were introduced into tobacco or soybean protoplasts and their activities assessed by CAT enzyme assays. Results in Fig. 2A show that the addition of the 338 bp 35S fragment increased the level of CAT activity as compared to that expressed from the unaltered NOS promoter. The stimulated activity is equal to the level observed from the intact 35S promoter-driven CAT, which is generally 5-fold higher than NOS promoter-driven CAT activity in these assays. The 338 bp fragment was equally effective in soybean and tobacco protoplasts (also see Fig. 4B, lanes 1, 2, 7, 8). A control plasmid with the 35S promoter inverted (53S) next to the CAT gene showed only background CAT activity, indicating that the CAT activity is dependent upon the promoter present in the chimeric gene.

To extend our observation to a stably transformed system the same gene constructions, with a NOS-P-neomycin phosphotransferase II-NOS 3′ kanamycin selection marker added to the plasmids, were introduced into tobacco protoplasts. Following selection on kanamycin, 40 independent transformed calli carrying the same construction were pooled and assayed for CAT activity, to average out any variation between individual transformants. The same stimulation of CAT activity due to the presence of the 338 bp 35S fragment, that was observed in the transient expression system, was seen in stably transformed calli (Fig. 2B). In this experiment the 338 bp fragment was found to function equally well when placed in either orientation. Individual plants regenerated from independent calli containing the same gene construction showed variable CAT activities, but an average again indicated the same result observed for calli and transient protoplast expression (data not shown). To insure that the addition of the 35S fragment did not alter the transcription start site, the 5′ ends of the transcripts expressed in transgenic plants from the NOS promoter and from the NOS promoter with the 338 bp fragment insert, were compared by RNase protection of a riboprobe extending 5′ to the normal transcription start site and found to be identical (data not shown).

To determine whether the 338 bp 35S fragment directs expression from the NOS promoter equally throughout the plant, as previously observed for the intact 35S promoter, CAT activities were assayed in leaf 2, 4, 6, 8 (from the top of the plant), upper stem, lower stem, and root extracts of several plants carrying each chimeric promoter construction. Though there were slight variations in activities in some tis-

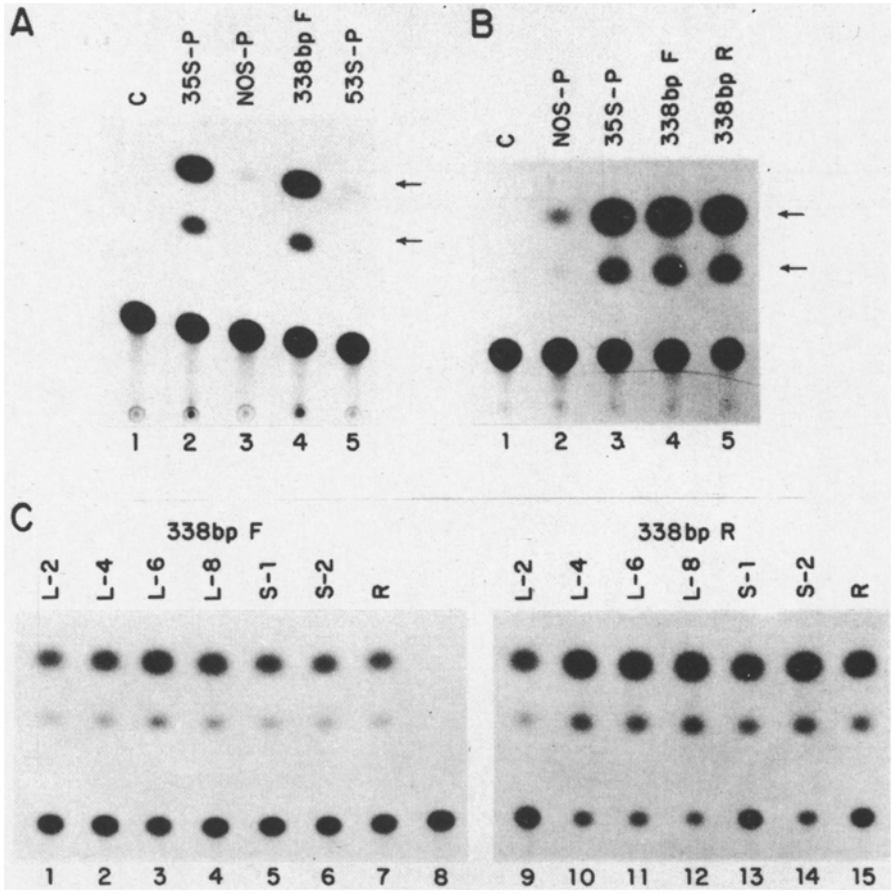

Fig. 2. (A) CAT enzyme assays on soybean protoplast transient expression extracts. Arrows mark the reaction products. Protoplasts received plasmids containing the CAT gene with the following promoter constructions: Lane 1) carrier DNA only; 2) 35S-P; 3) NOS-P; 4) NOS-P with 338 bp fragment in forward orientation at −150; 5) 35S-P in reverse orientation (53S). (B) CAT enzyme assays on transformed tobacco callus extracts. Callus is carrying the CAT gene with the following promoter constructions: Lane 1) carrier DNA only; 2) NOS-P; 3) 35S-P; 4) NOS-P with 338 bp fragment in forward orientation at −150; 5) NOS-P with 338 bp fragment in reverse orientation at −150. (C) CAT enzyme assays on transgenic tobacco plant tissues. Lanes 1−7 are samples from a plant carrying the CAT gene and the NOS-P with the 338 bp fragment inserted in the forward orientation at −150. Lanes 9−15 are samples from a plant carrying the CAT gene and the NOS-P with the 338 bp fragment inserted in the reverse orientation at −150. Samples are from leaf 2, 4, 6, 8, upper stem (S-1), lower stem (S-2), and root as labeled. Lane 8 is a control untransformed tobacco leaf extract.

sue extracts, the CAT activity level was generally similar thoughout each plant. CAT activities in representative plants are shown in Fig. 2C.

Tests for enhancer properties

The 338 bp fragment shown above to be equally effective in either orientation in stably transformed tobacco cells, was also equally active in both orientations when assayed in tobacco protoplast transient assays (Fig. 4C, lanes 5−8). Since orientation independence is one property of enhancers, we tested this fragment for other enhancer properties by placing it in other positions with respect to the NOS transcription start site. The fragment was cloned in sites located at −580, −740, −1200, +1000, and +2300 (Fig. 3A). Since these constructions are present in a circular plasmid, each site also has the following position: +6030, +5870, +5410, −5610,

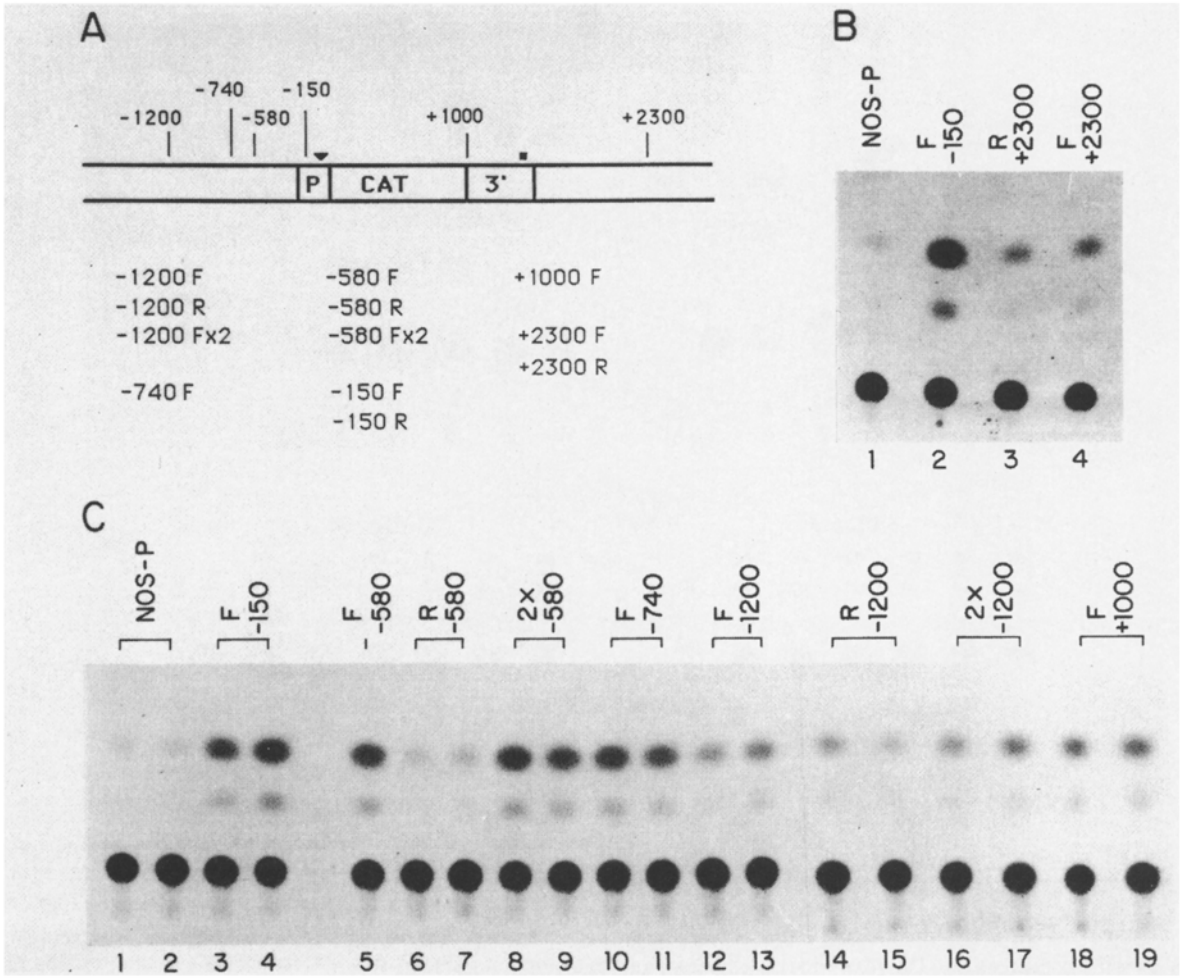

Fig. 3. (A) Diagram of the CAT chimeric gene showing the different sites where the 338 bp fragment was inserted. P stands for the NOS-P and 3′ stands for NOS 3′. The triangle marks the transcription start site for the NOS promoter. The square marks the polyadenylation site. Below are listed the insertions tested: F = forward insert, R = reverse insert, ×2 = double insert. (The doubles have both fragments in the same orientation.) (B) CAT enzyme assays on tobacco protoplast transient expression extracts. Protoplasts received plasmids containing the NOS-P-CAT gene with the 338 bp fragment inserted in the following locations: Lanes 1) no insert; 2) forward at −150; 3) reverse at +2300; 4) forward at +2300. (C) CAT enzyme assays on tobacco protoplast transient expression extracts. The percent of the total ^{14}C-chloramphenicol that is present in the acetylated products is given for each CAT assay sample. Protoplasts received plasmids containing the NOS-P-CAT gene with the 338 bp fragment inserted in the following locations: Lanes 1, 2) no insert: 4%, 4%; 3, 4) forward at −150: 15%, 18%; 5) forward at −580: 17.5%; 6, 7) reverse at −580: 7.5%, 6.5%; 8, 9) double forward at −580: 19%, 16.5%; 10, 11) forward at −740: 15.5%, 13%; 12, 13) forward at −1200: 8.5%, 9.5%; 14, 15) reverse at −1200: 8%, 7%; 16, 17) double reverse at −1200: 8%, 10%; 18, 19) forward at +1000: 10%, 12%.

and −4310, respectively. These more distant positions probably play no role due to the following results. At −580 the fragment is still fully active in the forward orientation, but has only two-fold stimulation in the reverse orientation (Fig. 3C). Two copies of the insert, both in the forward orientation, do not increase the activity over that seen with only one forward-oriented copy. The fragment has a 3.5-fold stimulation at −740, and only 2.5-fold stimulation at −1200. The reverse orientation at −1200 has a 2-fold stimulation. Double reverse fragments at −1200 show a slight increase over a single reverse insert, up to 2.5-fold stimulation, the same level of stimulation as from the single forward insert.

At +1000 the stimulation level is 3-fold, and at +2300 the stimulation remains detectable, but low (Fig. 3B). The orientation of the chimeric CAT gene within the plasmid had no effect on its level of expression as tested for the NOS-CAT-NOS 3′ construction and the NOS-CAT-NOS 3′ construction with the 338 bp insert at −150 (data not shown).

Sequences involved in stimulation activity

Fragments smaller than the 338 bp fragment used in the above studies were tested for their abilities to stimulate the NOS promoter in attempts to more specifically define the active sequence. Effects of a 303 bp fragment extending from −392 to −90 and a 76 bp fragment extending from −130 to −55 (see Fig. 4A), each located at −150 of the NOS promoter, were assessed in transient CAT assays. The 303 bp fragment in the forward orientation has a stimulation reduced to 3-fold, while in the reverse orientation its activity is further reduced to 1.5-fold (Fig. 4C). The stimulation level of the 76 bp fragment is 2-fold, with the reverse orientation down to 1.5-fold, and there is little effect of duplication.

To determine whether the 2-fold reduction in activity observed between the 338 bp (−392 to −55) and 303 bp (−392 to −90) fragments can be accounted for by sequences downstream from −90, a 5′ deletion of the 35S promoter that retains the sequences to −90 (Fig. 4A) was assayed. This deleted 35S promoter has only the activity level of the NOS promoter (Fig. 4C) suggesting that the region between −90 and −55 by itself does not raise the level of activity, but rather acts in conjunction with sequences 5′ to −90.

As a direct assay of the possible function of the SV40 "core enhancer" sequence that lies between −106 and −94 in the 35S promoter, a 182 bp fragment containing both copies of the SV40 72 bp repeat enhancer (−270 to −89 with respect to the SV40 early promoter transcription start site) and therefore including 2 copies of the SV40 core enhancer, was placed at −150 of the NOS promoter. This insert did not stimulate the NOS promoter as assayed by CAT activity in either tobacco or soybean protoplasts (Fig. 4B).

Discussion

The 35S promoter is a viral promoter with very high activity in a wide range of heterologous systems, and we have found that it contains a sequence able to stimulate transcription from a heterologous promoter. Properties of a 338 bp fragment of this promoter are enhancer-like and also show similarities to those described for the upstream activation sites (UASs) of yeast genes [8]. The fragment is more sensitive to orientation and position than the prototype enhancer example: the SV40 enhancer. The 338 bp fragment from the region 5′ to the 35S-P TATA box can stimulate expression of the heterologous NOS promoter up to the level of the full, intact 35S promoter and it is effective when placed in both orientations at −150. This activity was observed in both tobacco and soybean protoplasts, and in transgenic tobacco plants where the 338 bp fragment directed the same pattern of non-specificity in plant organ expression as does the intact 35S promoter. The normal transcription start site for the NOS promoter was not altered by the addition of the 35S fragment. The ability of this fragment to function, however, is very sensitive to position, showing a reduction in activity when moved further 5′ or placed 3′ to the coding region. Even at −580 it loses its orientation independence. The much lower activity of the fragment in reverse at −580, when the forward orientation is fully active at this location and the reverse orientation is fully active at −150, suggests that a functional region near the 3′ end of this fragment cannot act fully over the greater distance created by inversion.

The low activity of the inverted fragment at −580 argues against the possibility that the stimulation of the NOS promoter could be due to a distancing of negative regulatory sequences present in the NOS promoter or adjacent plasmid DNA. In this reverse construction all sequences are distanced the same as in the forward, highly active construction. Also the inactivity of the SV40 insert at −150 supports the conclusion that it is specifically the 35S fragment sequence that stimulates the NOS promoter expression. Results reported by Ellis *et al.* [2] during the preparation of this manuscript show that a 35S promoter region fragment, which is equivalent to our

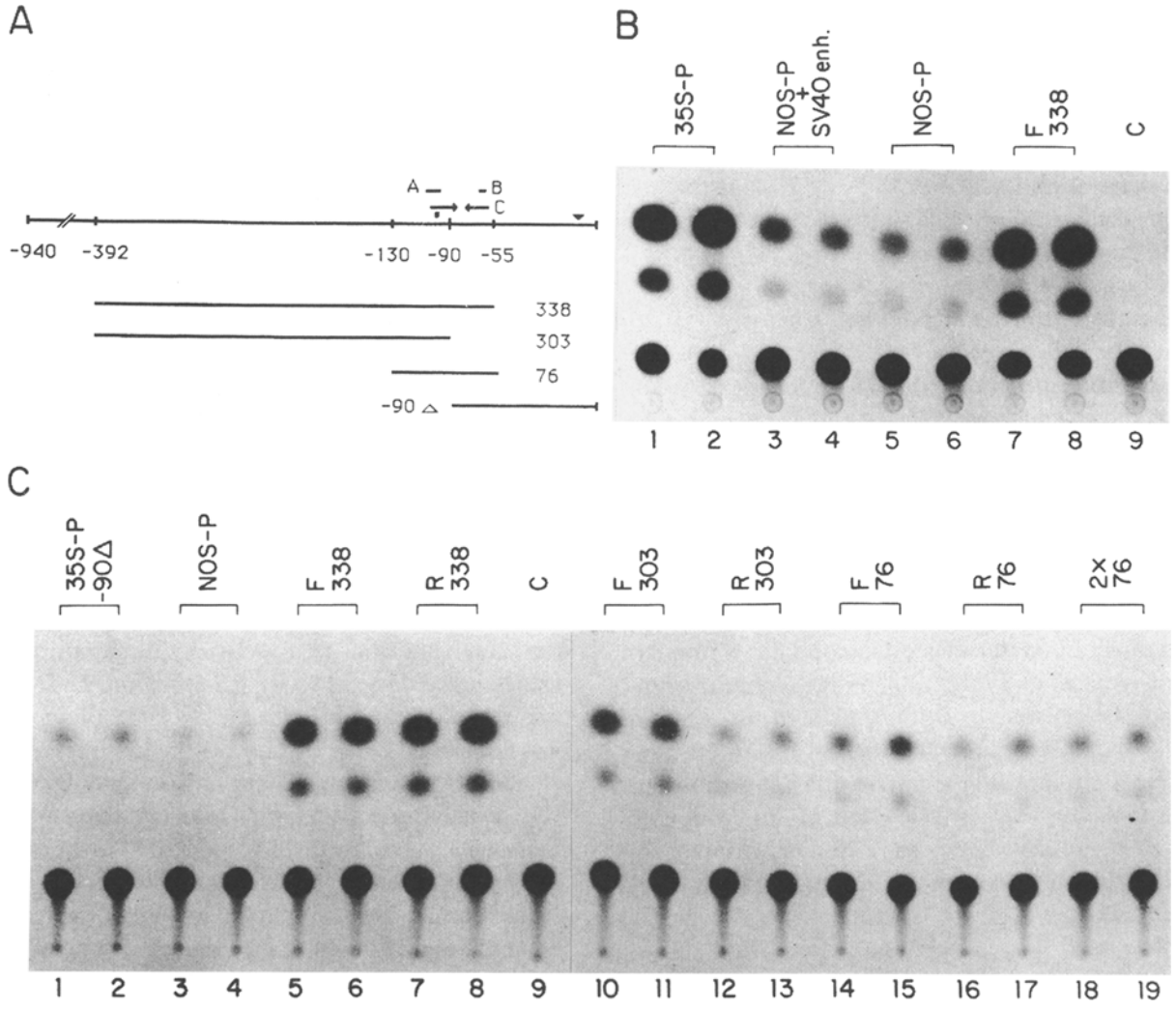

Fig. 4. (A) Diagram of the 35S promoter region showing the different fragments tested for transcription stimulation activity. Line A marks the SV40 core enhancer homology sequence, line B marks the CAT box sequence, and lines C mark the inverted repeat sequence. The triangle marks the transcription start site and the square marks the translation termination codon for the upstream gene VI coding region. (B) CAT enzyme assays on tobacco protoplast transient expression extracts. Protoplasts received plasmids containing the CAT gene with the following promoters: Lanes 1, 2) 35S-P; 3, 4) NOS-P with the SV40 enhancer inserted at −150; 5, 6) NOS-P; 7, 8) NOS-P with the 338 bp fragment inserted in the forward orientation at −150; 9) carrier DNA only. (C) CAT enzyme assays on tobacco protoplast transient expression extracts. The percent of the total ^{14}C-chloramphenicol that is present in the acetylated products is given for each CAT assay sample. Protoplasts received plasmids containing the NOS-P-CAT gene with the following fragments inserted at −150: Lanes 1, 2) not the NOS-P but a −90 deletion of the 35S-P: 5%, 4.5%; 3, 4) no insert in NOS-P: 4%, 4%; 5, 6) 338 bp in the forward orientation: 18%, 17%; 7, 8) 338 bp in the reverse orientation: 23%, 23%; 9) carrier DNA only: 1%; 10, 11) 303 bp in the forward orientation: 14%, 13%; 12, 13) 303 bp in the reverse orientation: 5%, 6%; 14, 15) 76 bp in the forward orientation: 6%, 8%; 16, 17) 76 bp in the reverse orientation: 5%, 6%; 18, 19) 2 copies of 76 bp: 5%, 7%.

303 bp fragment, can raise the expression of the maize alcohol dehydrogenase gene promoter in tobacco cells when placed at −140. This result provides an example of a second heterologous promoter that responds to sequences of the 35S promoter. They also reported that a fragment from the upstream promoter region of the octopine synthase gene can stimulate expression of the alcohol de-

hydrogenase promoter when placed in either orientation at −140. It will be interesting to see whether this transcription stimulating fragment has the same limitations that we have found for the 35S transcription stimulating fragment.

Fragments tested that are smaller than the 338 bp fragment have reduced activity indicating the removal of sequences required for maximum function. Since the 76 bp fragment that includes the SV40 "core enhancer" sequence, inverted repeat, and CAT box sequence has reduced activity, sequences 5′ to −130 are also functional. This result is in agreement with the previous deletion analysis [18] and exemplifies the compact nature of the CaMV genome. The coding region that lies upstream of the 35S transcription start site ends at −97 [4] and therefore must overlap the active 35S promoter sequence by over 33 bp, indicating a dual-function DNA sequence.

The removal of 35 bp from the 3′ end of the 338 bp fragment, which disrupts the inverted repeat (−103 to −57) and deletes the CAT box but retains the "core enhancer" sequence, reduces activity. This result, together with the finding that the −90 deletion retaining the CAT box but only a portion of the inverted repeat does not provide the lost increment of activity, suggests that the intact inverted repeat does play a role in transcription stimulation. Our finding that the SV40 enhancer, which includes two "core enhancer" sequences, is inactive in tobacco and soybean protoplasts indicates that the "core enhancer" sequence alone cannot stimulate transcription in plant cells. Since the "core enhancer" sequence lies mainly within one arm of the inverted repeat, its function may relate to other sequences, possibly the other arm of the inverted repeat. This type of relationship of the "core enhancer" sequence in the SV40 enhancer, with other sequences within the enhancer region, has been shown to occur [26]. The SV40 "core enhancer" sequence is necessary but not sufficient for function of the SV40 enhancer in animal cells.

The protoplast transient expression system was used for these assays to eliminate the problem of variability in expression of the same construction that has been observed between independent stable transformants [10, 16]. The 35S promoter has been shown to be highly susceptible to this phenomenon [19]. The low variability in our duplicate experimental samples establishes the reliability of our transient assay system. We did find a smaller differential in the activities of the 35S and NOS promoters in this system than has been described in stable transformation systems [15, 22]. This observation could be explained by a limitation of transcription or translation factors in our protoplasts, but the relative levels of expression of our promoter constructions would not be affected. The inability of multiple copies of stimulating fragments to raise expression above that directed by a single copy cannot be due to limiting factors, since the expression level in the cases of the 303 bp and 76 bp fragments is well below that from the intact 35S promoter. During the preparation of this manuscript Kay *et al.* [11] reported that duplication of a region in the 35S promoter that is equivalent to our 303 bp fragment did increase expression from the 35S promoter in stable transformants. Whether this conflicting result is due to their use of a homologous promoter system versus our heterologous promoter system, or to the assay system: stable versus transient, will need to be resolved.

To determine whether the transcription-stimulation activity of the 35S promoter upstream fragment is due to the binding of a trans-activating factor, we are now carrying out competition experiments using the transient protoplast assay system. Protein binding studies that we are undertaking will detect DNA sequences involved in protein interactions, and we expect these to include the inverted repeat sequence as well as a sequence 5′ to −130. In summary, the 338 bp fragment residing between −392 and −55 with respect to the 35S transcription start site can be used to raise the level of expression of a heterologous promoter up to the high level expressed from the intact 35S promoter. However, this fragment is sensitive to position and must be located within about 500 bp of the transcription start site for full activity. The cloning manipulations required to add this 35S transcription-stimulating fragment to a heterologous promoter will generally be more simple than those required to exchange a gene's endogenous promoter with the entire 35S promoter, making this system useful for increasing the expression of promoters or coding regions under investigation.

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References

1. Ecker JR, Davis RW: Inhibition of gene expression in plant cells by expression of antisense RNA. Proc Natl Acad Sci USA 83: 5372–5376 (1986).
2. Ellis JG, Llewellyn DJ, Dennis ES, Peacock WJ: Maize Adh-1 promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. EMBO J 6: 11–16 (1987).
3. Fluhr R, Kuhlemeier C, Nagy F, Chua N-H: Organ-specific and light-induced expression of plant genes. Science 232: 1106–1112 (1986).
4. Franck A, Guilley H, Jonard G, Richards K, Hirth L: Nucleotide sequence of cauliflower mosaic virus DNA. Cell 21: 285–294 (1980).
5. Fromm ME, Taylor LP, Walbot V: Stable transformation of maize after gene transfer by electroporation. Nature 319: 791–793 (1986).
6. Gorman CM, Moffat LF, Howard BH: Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol and Cell Biol 2: 1044–1051 (1982).
7. Gluzman Y, Shenk T (eds): Enhancers and Eukaryotic Gene Expression. Current Communications in Molecular Biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1983).
8. Guarente L, Hoar E: Upstream activation sites of the *CYC1* gene of *Saccharomyces cerevisiae* are active when inverted but not when placed downstream of the "TATA box". Proc Natl Acad Sci USA 81: 7860–7864 (1984).
9. Herrera-Estrella L, Depicker A, Van Montagu M, Schell J: Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303: 209–213 (1983).
10. Jones JDG, Dunsmuir P, Bedbrook J: High level expression of introduced chimaeric genes in regenerated transformed plants. EMBO J 4: 2411–2418 (1985).
11. Kay R, Chan A, Daly M, McPherson J: Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299–1302 (1987).
12. Khoury G, Gruss P: Enhancer elements. Cell 33: 313–314 (1983).
13. Krens FA, Molendijk L, Wullems GJ, Schilperoort RA: *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. Nature 296: 72–75 (1982).
14. Lin W, Odell JT, Schreiner RM: Soybean protoplast culture and direct gene uptake and expression by cultured soybean protoplasts. Plant Physiol 84: 856–861 (1987).
15. Nagy F, Odell JT, Morelli G, Chua N-H: Properties of expression of the 35S promoter from CaMV in transgenic tobacco plants. In: Zaitlin M, Day P, Hollaender A (eds) Biotechnology in Plant Science: Relevance to Agriculture in the Eighties. Academic Press, New York (1985) pp. 227–236.
16. Nagy F, Morelli G, Fraley RT, Rogers SG, Chua N-H: Photoregulated expression of a pea rbcS gene in leaves of transgenic plants. EMBO J 4: 3063–3068 (1985).
17. Nagy JI, Maliga P: Callus induction and plant regeneration from mesophyll protoplasts of *N. sylvestris*. Z Pflanzenphysiologie 78: 453–455 (1976).
18. Odell JT, Nagy F, Chua N-H: Identification of sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812 (1985).
19. Odell JT, Nagy F, Chua N-H: Variability of 35S promoter expression between independent transformants. In: McIntosh L, Key J (eds) Plant Genes and their Biology. Alan R. Liss, Inc. (in press).
20. Ou-Lee TM, Turgeon R, Wu R: Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplasts of rice, wheat, and sorghum. Proc Natl Acad Sci USA 83: 6815–6819 (1986).
21. Potrykus I, Shillito RD: Protoplasts: isolation, culture, plant regeneration. In: Weissbach A, Weissbach H (eds) Methods in Enzymology 118. Academic Press, New York (1985) pp. 549–578.
22. Sanders PR, Winter JA, Zarnason AR, Rogers SG, Fraley RT: Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Res 15: 1543–1558 (1987).
23. Shillito RD, Paszkowski J, Potrykus I: Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. Plant Cell Rep 2: 244–247 (1983).
24. Shillito RD, Saul MW, Pasylrowski J, Muller M, Potrykus I: High efficiency direct gene transfer to plants. Biotechnology 3: 1099–1103 (1985).
25. Timko MP, Kausch AP, Castresana C, Fassler J, Herrera-Estrella L, Van den Broeck G, Van Montagu M, Schell J, Cashmore AR: Light regulation of plant gene expression by an upstream enhancer-like element. Nature 318: 579–582 (1985).
26. Zenke M, Grundstrom T, Matthes H, Wintzerith M, Schatz C, Wildeman A, Chambon P: Multiple sequence motifs are involved in SV40 enhancer function. EMBO J 5: 387–397 (1986).