Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity

(eukaryotic promoter/promoter elements/plant virus/electroporation/luciferase)

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ABSTRACT   The cauliflower mosaic virus (CaMV) 35S RNA promoter has been dissected and examined in a transient expression system using the firefly luciferase gene as a reporter of promoter activity. Deletion analysis has shown that the 35S RNA promoter is composed of at least three regions—distal, medial, and proximal—which are essential for activity. The distal region contains three smaller elements homologous to the simian virus 40 "core" enhancer element, the medial region possesses a CCAAT-like box, and the proximal region contains a TATA box. A DNA segment encompassing the distal region is capable of activating the CaMV 35S core promoter in an orientation-independent, but not position-independent, fashion. The distal region can also activate a heterologous weak promoter, the CaMV 19S RNA promoter, albeit not to the high levels of the 35S RNA promoter. Multimers of the distal region are able to activate the 35S RNA promoter core to even greater levels of expression than the native 35S promoter. These experiments demonstrate that elements outside the boundaries of the core promoter (composed of proximal and medial elements) are recognized in a plant cell transient expression system.

Eukaryotic promoters that have been examined in detail are composed of multiple cis-acting elements, which are required for promoter function. These elements have been recognized in DNA manipulation experiments (deletions, mutagenesis, linker scanning analysis, element relocation, etc.) and bind trans-acting factors, which activate transcription (reviewed in refs. 1 and 2). Many, but not all, plant and animal promoters are composed of a TATA box, 25–30 bases upstream from the start of transcription, often a CCAAT box at about −80, and other elements that may confer greater or regulated activity on a promoter. The TATA box establishes polarity to the eukaryotic promoter; binds protein factors (3, 4); and alterations in sequence, orientation, or position dramatically reduce transcription altogether or from the normal start site (5). The CCAAT box, in promoters, in which it i,s found, is affected by alterations in sequence (5), appear,s in one instance (HV thymidine kinase promoter) in an inverted orientation with respect to the start of transcription (6), and binds distinct protein factors (7). Other upstream elements may differ from one promoter to another and presumably bind different trans-acting protein factors that may confer specialized functions, such as high level constitutive expression, tissue-specific expression, or response to environmental cues (reviewed in ref. 2). The elements that bind the transcriptional factor SP1 are well characterized and are found in a number of animal promoters, such as those in the 21-base-pair (bp) repeats of the simian virus 40 (SV40) early promoter (8, 9). Other upstream elements can act as

enhancers, such as those in the 72-bp repeats of the SV40 promoter—that is, they can function in a position- and orientation-independent fashion and are able to empower or regulate heterologous core promoters (10, 11).

A promoter that has been widely used in chimeric gene constructs in plants is the 35S RNA promoter from cauliflower mosaic virus (CaMV). This promoter drives high levels of RNA production in a wide variety of plants (12–18), including plants well outside the host range of the virus, such as monocots (19). In the context of the virus, the 35S RNA promoter is a strong promoter, and necessarily so, because it drives the synthesis of an RNA that serves as a nonreusable template for CaMV DNA synthesis (20, 21). CaMV also has another promoter, the 19S RNA promoter, which drives the synthesis of an mRNA that encodes the most abundant viral-encoded protein (22, 23).

In this paper, we have undertaken a further study of the 35S RNA promoter using the firefly luciferase cDNA as a "reporter" of promoter activity in transient expression assays (24). Luciferase catalyzes the oxidative decarboxylation of luciferin and in the process produces light (25). The firefly luciferase assay is rapid, inexpensive, sensitive, and produces quantitative results (26). The luciferase assay is highly sensitive because luciferase converts chemical energy to light with high quantum efficiency (27).

METHODS

5′-End Deletions of the CaMV 35S RNA Promoter. Plasmid pDO432 has the firefly luciferase coding region inserted between an upstream DNA segment containing the CaMV 35S RNA promoter (−1585 to +1, relative to the start of transcription) and a downstream fragment containing the nopaline synthase polyadenylylation signal site (24). The unique *Acc* I site (−390) was used as the starting site for BAL-31 digestion and *Xho* I linkers were ligated to deletion end points with T4 DNA ligase. To bring the deletion end points into juxtaposition with common flanking sequences, the BAL-31-digested plasmids were cleaved with *Xho* I and *Hin*dIII (site at −1585), the staggered ends were filled in with DNA polymerase I (Klenow fragment), and plasmid DNAs were closed with T4 ligase and used to transform *Escherichia coli* HB101. Blunt-end joining between *Xho* I and *Hin*dIII sites restored the *Hin*dIII site, which permitted rapid screening of plasmids for desired deletion end points. Deletion end points, which all join onto the *Hin*dIII site of the pUC19 plasmid backbone, were determined by dideoxynucleotide

sequencing using a reverse-sequencing primer (New England Biolabs, Beverly, MA) on a double-stranded DNA template.

Reorientation or Translocation of the 35S RNA Promoter Distal Region. To access the 35S RNA promoter distal region (DR), pJO62, progenitor to pJO62d and containing an *Xho* I linker at −148, was cleaved with *Eco*RV (−89) and reclosed with the addition of a *Sal* I linker. Subsequently, the DR segment from *Xho* I (−148) to *Sal* I (−89) was gel-purified and ligated at the *Xho* I site of truncated 35S RNA promoters pJO44d (−73), pJO4x (−89), pDO478 (−115), and pDO479 (−380). To move the DR segment further upstream from the −89 promoter, an 850-bp *NPT-II* gene fragment was inserted via *Sal* I linkers into the *Eco*RV site (−89) of pJO62d to create pDO625. The isogenic plasmid (pDO625) without the DR segment has the *NPT-II* gene inserted at an *Xho* I linker at −89 of pJO4x. To place the DR segment downstream from the luciferase gene, a *Sal* I linker was first introduced into the *Kpn* I site of pJO4x (≈440 bp downstream from the polyadenylylation site in the *nos* 3' segment) to create pDO606. Subsequently, the DR segment was inserted into the *Sal* I site of pDO606.

Fusion of the Luciferase Coding Region to the CaMV 19S RNA Promoter. The CaMV 19S RNA promoter was fused to the luciferase coding region by replacing the CaMV 35S RNA promoter fragment in pDO432 with either a 126- or a 391-bp 19S RNA promoter fragment from pLW414 (28). The 19S RNA promoter fragment extending to +11 was accessed on the downstream side by inserting a *Sal* I linker at an *Sph* I site created by oligonucleotide mutagenesis at position 5772 on the CaMV genome map in pCaMV10 (G. Baughman, personal communication). The upstream side of the 19S RNA promoter was accessed by introduction of an *Xho* I linker at a *Pst* I site (−380; map position 5383) or at an *Eco*RI site (−115; map position 5646).

Transient Expression Assay. Protoplasts from *Daucus carota* suspension cell line W001C (from R. Sung, University of California Berkeley) were transfected with CsCl gradient-purified plasmid DNA using an electroporation procedure modified from Fromm *et al.* (19). Plasmid DNA (10–15 μg) and sonicated calf thymus DNA (500 μg) as carrier were mixed with $0.5–1 \times 10^7$ protoplasts in a final buffer vol of 1.2 ml and subjected to a 275-V 100-msec pulse discharged from a 320-μF capacitor. Cell extracts were prepared 18–20 hr after electroporation by three cycles of freezing–thawing in 500 μl of extraction buffer (100 mM potassium phosphate buffer, pH 7.5/1 mM dithiothreitol) followed by 3 sec of sonication. The extracts were clarified by centrifugation for 3 min at 4°C in a microcentrifuge. Luciferase activity was determined by mixing 0.05–0.1 vol (25–50 μl) of the supernatant fluid with 100 μl of luciferase assay buffer (36 mM glycylglycine, pH 7.8/20 mM $MgCl_2$/12 mM ATP/1 mg of bovine serum albumin per ml) and initiating the reaction by injecting 100 μl of 0.4 mM luciferin into the mix. Peak light intensity was measured in a luminometer (LKB, model 1250) connected to a chart recorder.

RESULTS

Structure of the CaMV 35S RNA Promoter. To define functional elements within the CaMV 35S promoter, we carried out a 5'-end deletion analysis on a DNA fragment from the CaMV genome extending 1.6 kilobases upstream from the 35S RNA cap site (29). The start site of 35S RNA transcription (C*GACAC*, start site italicized) was accessed by altering the site, using oligonucleotide-directed mutagenesis, to a *Bam*HI site, GGATCC (G. Baughman, personal communication). The 35S promoter fragment was joined to the firefly luciferase cDNA at a *Bam*HI site 80 bases upstream from the start of luciferase translation. In preliminary experiments, we found that the region extending 400

Table 1. Relative activity of 5' deletions of the CaMV 35S RNA promoter

Plasmid	Deletion end point	Relative activity	SD
pDO432	−1600	115	18
pJO6d	−365	114	16
pJO4d	−349	107	26
pJO24d	−302	88	13
pJO14d	−248	81	14
pJO34d	−223	93	12
pJO62d	−148	100	16
pJO382	−134	91	21
pJO398	−108	14	5
pJO396	−104	18	4
pJO4x	−89	23	7
pJO44d	−73	0.8	0.2
pJO48d	−68	0.8	0.1

Relative luciferase activity, normalized to pJO62d, is the mean of six or more samples from at least three independent experiments. SD is the population standard deviation.

bases upstream from the start of transcription to the *Acc* I site was as active in transient expression assays as the full 1.6-kilobase fragment. Therefore, we initiated 5' deletions with BAL-31 digestion from the *Acc* I site.

Deleted constructs were introduced into carrot cells by electroporation, and it was found that deletions with end points prior or up to position −148 (with respect to the cap site) retained nearly full activity in transient assays (Table 1). Beyond −148, more closely approaching the promoter, activity dropped significantly at two points (Fig. 1). Promoter activity dropped sharply to only 20% of full activity in deletions with end points beyond −134, and in deletions beyond −89, promoter activity dropped precipitously to only 0.8% of full activity.

When the activity profile of the promoter deletions was superimposed on the sequence of the 35S RNA promoter, the first drop in activity occurred in an upstream region, the DR (Fig. 2), that contains three elements homologous to the SV40 "core" enhancer, $GTGG\overset{AAA}{TTT}G$ (30). Of the three elements in the DR, the two outer ones are oriented in the forward direction and the middle one is in the reverse direction. Surprisingly, deletions to −134, which eliminated the 5'-most element in this domain, had no detectable effect on promoter activity, but deletions to −108, eliminating the second element oriented in the opposite direction, had a profound effect on promoter activity.
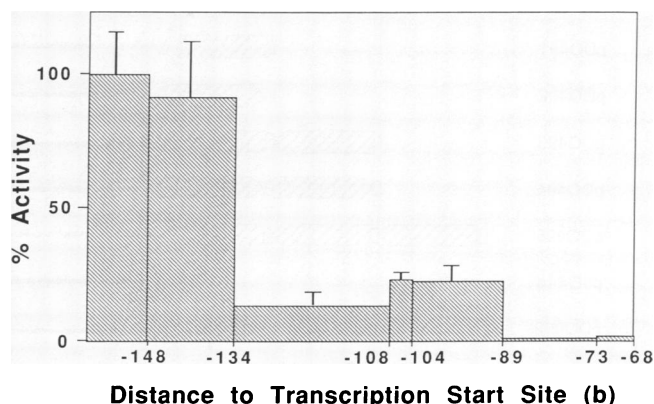

Distance to Transcription Start Site (b)

FIG. 1. 5'-End deletion analysis through the DR and MR of the CaMV 35S RNA promoter. Relative mean values and population SDs for luciferase activity are normalized to pJO62d as described in Table 1. DNA sequence for this region is shown in Fig. 2. b, Bases.
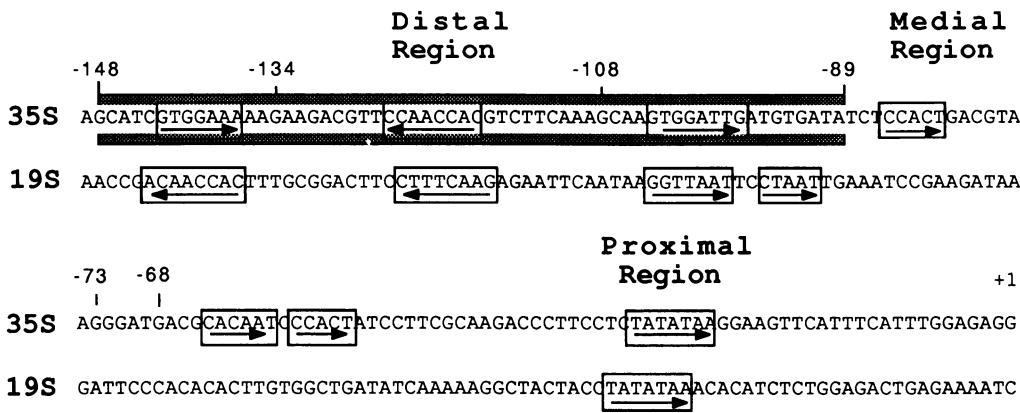
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                    Distal                                    Medial
                    Region                                    Region
         -148           -134                    -108              -89
          |              |                       |                |
35S   AGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTA

19S   AACCCACAACCACTTTGCGGACTTCCTTTCAAGAGAATTCAATAAGGTTAATTCCTAATTGAAATCCGAAGATAA
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                                            Proximal
         -73  -68                            Region                              +1
          |    |
35S   AGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGG

19S   GATTCCCACACACTTGTGGCTGATATCAAAAAGGCTACTACCTATATAAACACATCTCTGGAGACTGAGAAAATC
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FIG. 2. Comparison of the CaMV 35S and 19S RNA promoters. The DR DNA segment (−148 to −89) is highlighted in a stippled box. Sequences homologous to the SV40 core enhancer, CCAAT, and TATA elements are boxed.

The second and most precipitous drop (by a factor of 30) in activity occurred in a region that we call the medial region (MR). The MR contains a CCAAT-like box at an expected position (−85) and two other CCAAT-like boxes closer to the start of transcription (Fig. 2). Promoter activity plummeted to barely detectable levels when the DNA fragment containing the 5′-most CCAAT element was deleted from the promoter (as in pJO44d). The 5′-end deletions of the downstream CCAAT-like boxes had little further effect on expression. The remaining functional region closest to the start of transcription, the proximal region (PR), contains a TATA box at −31. The functionality of the PR has not been tested here but has been demonstrated by others (31) in chimeric constructs with other promoter elements.

DR Can Activate the 35S RNA Core Promoter. We tested whether the DR can function in a position- and orientation-independent fashion and whether it endows greater transcriptional activity on a heterologous promoter. To do so, a DNA segment containing the DR (−148 to −89) was excised, linkers were attached, and the DR segment was reinserted in the same position (−89). Removal of the DR segment reduced the activity of the 35S promoter by a factor of ≈5 (compare pJO62d to pJO4x in Fig. 3), and reinsertion of the DR segment restored ≈60% of full activity. Reinsertion of the DR segment in the same orientation, however, did not recreate the native promoter, because an 8-bp linker was interposed between the

DR and MR in pJO4x. Either the sequence or the spacing change brought about by presence of the linker apparently reduced the activity of the promoter. Unexpectedly, when the DR segment was reinserted in the opposite orientation (pDO492), full activity of the promoter was restored.

To determine whether activation effects of the DR segment were additive, multiple tandem copies of the DR segment were attached to the −89 promoter core containing MR and PR elements. Interestingly, the activity of the 35S promoter increased in almost exact proportion to the number of DR segments (compare pJO4x, pDO491, pDO493, and pDO495 in Fig. 3). With three DR segments attached, the promoter is nearly twice as active as the 35S minimal promoter (pJO62d). However, two copies of the DR segment in opposite orientation (pDO494) gave no greater activity than one copy in a similar orientation (pDO492).

In the 5′-end deletion analysis, it was shown that removal of the DNA segment containing the CCAAT-like box at −85 in the MR produced the most profound effects on the function of the 35S promoter. To determine whether the CCAAT box-containing segment was dispensable to the promoter core in the presence of the DR segment, we attached the DR segment to the −73 promoter in pJO44d (Fig. 3). The result was pDO602, which, in effect, is an internal deletion from −89 to −73, removing the −85 CCAAT-like box and replacing it with an 8-bp linker. pDO602 with the DR segment

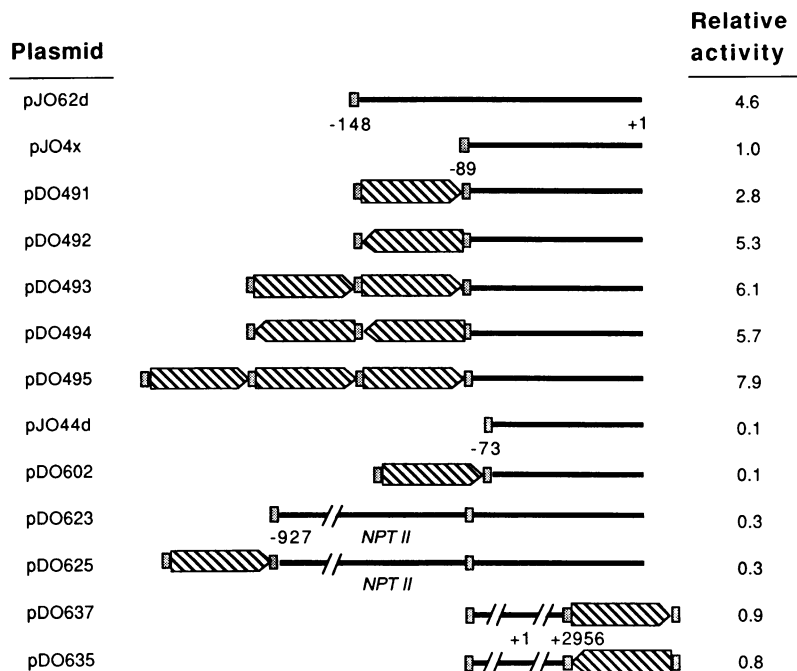| Plasmid		Relative activity
pJO62d		4.6
pJO4x	-148 to +1	1.0
pDO491	-89	2.8
pDO492		5.3
pDO493		6.1
pDO494		5.7
pDO495		7.9
pJO44d		0.1
pDO602	-73	0.1
pDO623	-927 NPT II	0.3
pDO625	NPT II	0.3
pDO637		0.9
pDO635	+1 +2956	0.8

FIG. 3. Inversion and duplication of the CaMV 35S RNA promoter DR segment (−148 to −89) flanked by *Xho* I and *Sal* I linkers (hatched arrows flanked by small rectangles). Relative activity, normalized to pJO4x, is the mean of six or more samples from at least three independent experiments.

attached was quite inactive, with no more promoter activity than pJO44d, suggesting that the DR segment cannot supply or substitute for promoter core functions (e.g., CCAAT box functions) and can only activate a complete promoter core.

To determine whether the effects of the DR segment are influenced by position, the DR segment was moved a considerable distance upstream and downstream from the −89 promoter. The DR segment was moved upstream to −927 by inserting the ≈840-bp bacterial *NPT-II* at the −89 site (pDO623; Fig. 3). From the upstream position, the DR segment (at one copy) was ineffective in stimulating residual promoter activity when compared to the isogenic plasmid (pDO623) bearing the *NPT-II* gene but missing the DR segment. It should be pointed out that for unexplained reasons pDO623 was less active than the control plasmid (pJO4x) lacking the *NPT-II* gene. The DR segment was also moved a considerable distance downstream to +2956, ≈440 bp downstream from the polyadenylylation site in the *nos* 3′ segment (Fig. 3). The DR segment in the downstream position failed to stimulate the 35S RNA core promoter in either the forward (pDO637) or reverse direction (pDO635). Thus, the DR segment appears to have positional constraints in that one DR copy does not stimulate the 35S RNA promoter core from a remote site on the same plasmid.

DR Can Activate a Heterologous Promoter. To determine whether the 35S promoter DR segment can empower a heterologous promoter, the DR segment was placed upstream from truncated forms of the 19S promoter. The 19S promoter was joined to the luciferase–*nos* 3′ reporter gene via a *Sal* I linker inserted just before the start of translation of ORF-VI, 11 bases downstream from the 19S cap site. The 19S promoter fragment with 390 bp upstream from the start site of transcription (pDO479; Fig. 4) was 80 times less active than the minimal 35S promoter (pDO148), and a truncated form with 110 bases upstream from the start of transcription (pDO478) was 150 times less active than the 35S promoter.

When one copy of the 35S RNA promoter DR segment was appended onto the 19S promoter truncated to position −110 (as in pDO488), promoter activity more than doubled over the basal level of the promoter core (pDO478). When additional DR segments were added in tandem (as in pDO498 and pDO600), activity increased nearly in proportion to the number of DR segments added. When one copy of the 35S promoter DR segment was appended onto the larger 19S promoter fragment with a deletion end point at position −390 (as in pDO489), promoter activity was enhanced only 1.4-fold. Additional copies of the 35S promoter DR segment at position −390 further stimulated the 19S promoter (as in pDO499 and pDO601), but less so than when the DR segment was appended onto the truncated promoter at position −110. Thus, the 35S promoter DR segment can activate a heterolo-

gous promoter, but cannot stimulate luciferase expression from the 19S RNA promoter core to a level comparable to that driven by the normal 35S RNA promoter.

DISCUSSION

The CaMV 35S RNA promoter is a composite structure residing in the 150-bp DNA segment upstream from the start of 35S RNA transcription. The 35S promoter appears to have at least three functional domains—a proximal region (PR) composed of a TATA box, a MR containing a CCAAT-like box element, and a DR with three elements homologous to the SV40 core enhancer, $GTGG\overset{AAA}{TTT}G$ (30). The promoter deletion analysis reported here demonstrates the essential role of elements within the DR and MR on expression of the 35S RNA promoter. We purposely selected deletions with end points that flank elements with homology to other known promoter elements, such as the CCAAT box and the core enhancer element. However, given the resolution of the present analysis, we cannot, as yet, define the exact boundaries of the functional elements. Nonetheless, upstream regions of other plant genes possess elements similar to those we have pointed out in the 35S RNA promoter, such as sequences homologous to the SV40 core enhancer (32–39). However, the functional properties of these sequences have not been examined at high resolution.

Odell *et al.* (17) reported similar results in a deletion analysis of the 35S RNA promoter using larger deletions, except they found higher residual promoter activity (5%) in a PR fragment (−46/+9), which contained little more than the TATA box. In our experiments, even somewhat larger promoter fragments, such as a −79 promoter, had <1% of full activity. The difference may be that in the experiments by Odell *et al.* (17) the constructs were inserted into *Agrobacterium* Ti plasmid vectors and transferred into the plant genome. In such an environment, it is possible that heterologous flanking sequences might activate the proximal element of the 35S RNA promoter.

The 35S RNA promoter DR shares some, but not all, properties of elements that activate other promoters, such as enhancer elements in animal cell promoters (10, 11). For example, a segment containing the DR (−148 to −86) acts in an orientation-independent fashion when appended onto the 35S RNA promoter core, composed of MR and PR elements. However, unlike an enhancer, the activity of the DR was influenced by position, and a single copy of the DR was ineffective in stimulating the core promoter from a distant upstream or downstream site. It is possible, but remains to be tested, that distant effects could be seen with more copies of the DR. The addition of multiple DR segments at close range

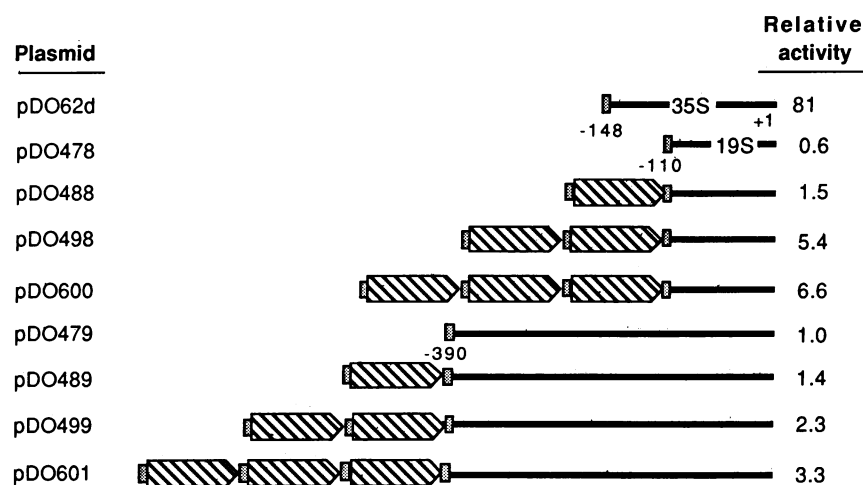| Plasmid		Relative activity
pDO62d	▯——35S———	81
	−148 +1	
pDO478	▯——19S—	0.6
	−110	
pDO488	▯▨▨▨▨▶▯——————	1.5
pDO498	▯▨▨▨▨▶◀▨▨▨▨▶▯——————	5.4
pDO600	▯▨▨▨▨▶◀▨▨▨▨▶◀▨▨▨▨▶▯——	6.6
pDO479	▯———————————————	1.0
	−390	
pDO489	▯▨▨▨▨▶▯———————	1.4
pDO499	▯▨▨▨▨▶◀▨▨▨▨▶▯—————	2.3
pDO601	▯▨▨▨▨▶◀▨▨▨▨▶◀▨▨▨▨▶▯———	3.3

FIG. 4. Fusion of the CaMV 35S RNA promoter DR segment (hatched arrows flanked by small rectangles) to the CaMV 19S RNA promoter. Relative activity is normalized to pDO479 and represents the mean value of six or more samples from at least three independent experiments.

to the 35S RNA promoter core was effective and additive as has been described for the SV40 enhancer (40) and the −89 promoter with three DR segments was nearly twice as strong as the native 35S promoter (Fig. 3).

Like other promoter-activating elements, the 35S RNA DR segment can also activate a heterologous promoter, as we have shown here with the 19S RNA promoter. The 19S RNA promoter core was, indeed, activated by the 35S promoter DR, but not to levels of the 35S promoter itself. In our transient expression system, the 19S RNA promoter was much less active than the 35S RNA promoter in driving luciferase expression. However, the strength of the two promoters as assessed by luciferase production may not be directly comparable, because in our constructs the leader sequences in the RNAs encoding luciferase from the two promoters were not identical (because the 19S RNA promoter end point is at +11, while the 35S RNA promoter end point is at +1). There are other possible explanations for the inability of the 35S RNA promoter DR segment to drive the 19S RNA promoter to 35S RNA promoter levels—one being that undetermined positional relationships between the DR segment and the 19S promoter core may be required for full activity. The subtleties of close positional relationships have been observed in the alignment of the 72-bp enhancer segment to the 21-bp repeats in the SV40 enhancer (41). However, another possibility is that the segment we have chosen as a 19S core promoter (composed of two CCAAT-like boxes and a TATA box; Fig. 2) may be a poor core promoter. On the other hand, the 35S RNA core promoter seems to function well in chimeric promoter constructs. For example, Fluhr *et al.* (31) have fused regulatory elements that respond to light to the −46 35S RNA core promoter and demonstrated the light-regulated expression of this fused promoter.

These experiments also demonstrate that a plant cell transient expression system responds to elements outside the promoter core (when the promoter core refers the MR and PR of the 35S RNA promoter). The presumed plant factors that recognize the 35S RNA promoter DR must be reasonably abundant in carrot cells, because the response of carrot cells to added DNA containing the 35S promoter is almost linear up to the highest concentrations we have tested (50 μg/ml). Furthermore, the essential elements of the 35S promoter are apparently recognized in a wide variety of plants well outside the host range of the virus. The 35S promoter is a strong promoter in tobacco, petunia, corn, and carrot cells (12–19) even though none of these plants is a host for CaMV.

Finally, the luciferase assay is a powerful tool for assessing gene expression. The assay is highly sensitive, permitting one to measure low levels of luciferase production. In our experiments, we have measured light emission over a range of 5–25,000 light units in extracts from 10^6 cells. Assuming 10^6 units of light from 3×10^{11} luciferase molecules (26), we have detected on the average as little as 1 luciferase molecule per cell. (This number is not corrected for the number of cells that actually express luciferase.) In addition to the greater sensitivity of the luciferase assay over the standard chloramphenicol acetyltransferase assay, the luciferase assay has no detectable background in extracts from plant cells. Furthermore, because the luciferase assay is rapid and inexpensive, multiple samples can be tested, as was done in this study, to obtain more quantitative data.

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