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Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays

(constitutive promoter/Z-DNA/Ti plasmid/transformation/electroporation)

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ABSTRACT **We studied the fine structure of the nopaline synthase (*nos*) promoter, which is active constitutively in a wide range of plant tissues, by both transient and stable transformation expression analyses. 3′ and 5′ deletion fragments were linked to form a set of internal deletion and duplication mutants that scanned the *nos* promoter. These mutated promoters were linked to the gene for the marker chloramphenicol acetyltransferase (CATase) as a means to readily assay promoter strength. The stable transformation analysis revealed the functional importance of an extended CCAAT box region (−97 to −63). Deletion of an upstream region (−112 to −101) containing an octameric repeated element resulted in a reduction in promoter strength by a factor of 30. A further deletion (−119 to −101) disrupted a potential Z-DNA-forming element as well, totally eliminating promoter function. Thus, a 19-base deletion across a repeated octamer and a potential Z-DNA-forming element identifies an essential upstream activator in the *nos* promoter. Duplication of the upstream element tripled promoter activity. Electroporation-mediated transient analysis was unable to distinguish downstream promoter elements. However, the upstream element behaved similarly in both assays in that deletion of the entire upstream element resulted in no promoter activity and that duplication of the element significantly enhanced the promoter strength.**

The *Agrobacterium* nopaline synthase gene (*nos*) is integrated as a part of the transferred DNA (T-DNA) to plant chromosomes after *Agrobacterium*-mediated plant transformation (1–3). Though the gene is of bacterial origin, it is readily expressed in plant tissues in a constitutive fashion. The small size (4) and constitutive nature (2, 3) of this promoter make it a useful tool for the analysis of gene expression in plants, analogous to the invaluable simian virus 40 and herpes simplex viral promoters, which have been used successfully in animal promoter studies (5–7).

Both TATA and CCAAT box homologies, which are found in many eukaryotic control regions, have been identified also in the *nos* promoter (8, 9) and shown to be functionally important (4, 10). It is poorly understood whether the constitutive *nos* promoter also carries other control elements such as positive activators, enhancers, or silencers (11–15). These elements are either essential for gene activity or modulate the activity of a functional promoter by providing binding sites for factors that induce or inhibit transcription initiation. Common promoter structural features that may overlap the above elements include repeating sequence motifs (5, 16) and alternating purine/pyrimidine blocks that may form a left-handed DNA helix (Z-DNA) (17, 18). In this study, we have examined the upstream sequences of the *nos* promoter in stably or transiently transformed plant cells to determine their involvement in gene expression.

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The use of transient assay systems involving electroporation of plant protoplasts is becoming increasingly widespread to analyze promoter function (19, 20). Thus, we deemed it important to examine the efficacy of the electroporation-mediated transient assay systems toward plant promoter analysis. Since the *nos* promoter is active constitutively in all of the tobacco tissues that have been examined (2, 3) and in isolated tobacco protoplasts (20), it is ideally suited to these studies. This paper provides both a detailed description of the *nos* promoter control elements and a comparison of stable and transient promoter analysis systems.

MATERIALS AND METHODS

Strains. *Escherichia coli* strain MC1000 (21) was used as the host for routine cloning experiments. *Agrobacterium* strain A281 (22), containing the C58 chromosomal background and the wild-type supervirulent plasmid pTiBo542, was used as the host for the binary vectors and for tobacco transformation. *Nicotiana tabaccum* cell suspension culture designated NT1 (23) was used for both stable transformation and electroporation experiments.

***Agrobacterium* Transformation.** *Agrobacterium* A281 cells were transformed with binary vector constructs by the freeze/thaw procedure (24–26). Briefly, 100 ml of YEP medium (1% yeast extract/1% peptone/0.5% NaCl, pH 7.5) was inoculated with 4 ml of a fresh overnight culture. The cells were grown for about 4 hr at 28°C with vigorous shaking (220 rpm) until the OD_{600} value reaches about 0.5–1.0. The cells were pelleted, resuspended in 2 ml of YEP medium, and divided into 100-μl aliquots on ice. About 0.1 μg of DNA was added, and the cells were frozen in liquid nitrogen and heat-shocked for 5 min at 37°C. One ml of YEP medium was added, and the cells were allowed to recover for 2–4 hr at 28°C with gentle shaking. Finally, the bacteria were plated on selective YEP agar medium containing 3 μg of tetracycline and 20 μg of kanamycin per ml and allowed to grow for 2–3 days at 28°C until transformed colonies appeared.

***Agrobacterium* Plasmid Quick-Screen.** The structure of binary Ti plasmids taken up by the *Agrobacterium* cells was analyzed by a quick-screen method (25) based on the *E. coli* alkaline lysis procedure (27). The cells were grown for at least 24 hr in YEP medium supplemented with the appropriate antibiotics. Cells (1.5 ml) were pelleted in an Eppendorf tube, resuspended in 100 μl of suspension buffer (10 mM EDTA/150 mM glucose/25 mM Tris·HCl, pH 8.0, to which 4 mg of lysozyme was added per ml just before use), and incubated for 10 min at room temperature. Fresh alkaline $NaDodSO_4$ solution (200 μl of 0.2 M NaOH/1% $NaDodSO_4$) was added, and the tubes were shaken. Thirty microliters of alkaline phenol (phenol equilibrated with 2 vol of 0.2 M NaOH) was added, and the cells were mixed in a Vortex extremely briefly (about 1 sec). The cells lysed instantaneously, and the

Abbreviations: bp, base pair; CATase, chloramphenicol acetyltransferase; T-DNA, transferred DNA.

suspension became very viscous; 150 μl of 3 M sodium acetate (pH 4.8) was added, and the tubes were shaken gently and left at −20°C for 15 min. The residue was then pelleted, and the DNA was precipitated twice, rinsed with 70% ethanol, and resuspended in 50 μl of TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0). Five microliters of the DNA was used for structural analysis by restriction enzyme digestion.

Plant Transformation. NT1 cells were stably transformed as described (23). After 3–4 weeks, 50 calli from each cocultivation were transferred to a fresh selective agar plate. The transformed calli were grown for another week and harvested for assay of chloramphenicol acetyltransferase (CATase).

Electroporation. Exponentially growing NT1 cells were protoplasted with cellulase and pectolyase (28), resuspended in electroporation buffer (18) at a concentration of 5×10^6 cells per ml, and left on ice. Test DNA (5 μg) and carrier DNA (45 μg) in 50 μl of water were mixed with 0.5 ml of protoplast suspension. The cells were electroporated by using a 210-μF capacitor at 385 V across a 1-cm path in a disposable microcuvette equipped with stainless steel electrodes. The electroporated cells were left on ice for about 20 min and then added to 8 ml of liquid MS medium (29) supplemented with 0.2 mg of 2,4-dichlorophenoxyacetic acid per liter and 0.4 M mannitol. The protoplasts were cultured in 100-mm Petri plates at 28°C for 19–20 hr and harvested for CATase activity analysis.

CATase Activity Determination. Protein was liberated by sonication and centrifugation for 4 min. CATase assays (30) were performed on 20 μg of total protein prepared from stably transformed cells incubated for 10 min or 240 μg of total protein from transiently transformed protoplasts incubated for 60 min. Promoter strength is expressed as the percentage wild-type activity after subtraction of the background level.

RESULTS

Physical Description of the *nos* Promoter. Many *nos* promoter features have been described (4, 8–10). The promoter carries the consensus TATA box and CCAAT box sequences at −26 and −78, respectively. There are two 11-base-pair (bp) repeat elements designated *a* and two 8-bp repeat elements designated *b* in alternating tandem array forming two *a*/*b* repeat pairs at the upstream region, both of which are three turns of the DNA helix in length (Fig. 1). Relevant to this study is the presence of a stretch of 10 alternating purine and pyrimidine residues (GCACATACGT) immediately upstream of the downstream *b* element. This sequence is a potential Z-DNA-forming element similar to those found in a number of other regulatory systems (15–17). No other potential Z-DNA-forming sequences were found elsewhere in the promoter. Thus, the downstream *a*/*b* repeat pair differs from the upstream pair primarily in that the sequence between the downstream *a* and *b* elements forms a potential Z-DNA-forming region and in that the orientation of the *b* element is inverted.

Plasmid Construction. We have constructed a set of internal deletion and duplication mutants (pGA561) by linking 3′ promoter deletion mutants (pGA355) to 5′ promoter mutants (pGA553) connected to the reporter gene for CATase on a binary vector (31) generated previously (4). All the mutants carry a 9-bp sequence (CCGGATCTG) at the fusion site generated by joining *Bam*HI and *Bgl* II linkers. The deletion endpoints are indicated in Fig. 1.

The plasmids were then transferred to *Agrobacterium* strain A281 carrying the supervirulent nondisarmed helper Ti plasmid pTiBo542 directly by the freeze/thaw transformation procedure. The direct transformation method is not only rapid but also reduces the plasmid rearrangement observed after triparental mating. This method results in about 10^3 transformants per μg of DNA. The structure of the transferred plasmids was verified by a quick-screen procedure that facilitates the rapid analysis of binary vectors directly from *Agrobacterium*. NT1 calli were transformed with *Agrobacterium* cells carrying various mutant *nos* promoters, and the promoter strength was measured as CATase activity (Table 1).

Identification of an Extended CCAAT Box Region and a Distinct Upstream Element. The CCAAT box deletions −81/−63 and −82/−63 caused CATase activity to drop to 24% and 29% of wild type, respectively (Table 1, group B). A further deletion, −97/−63, gave 5% of wild-type promoter

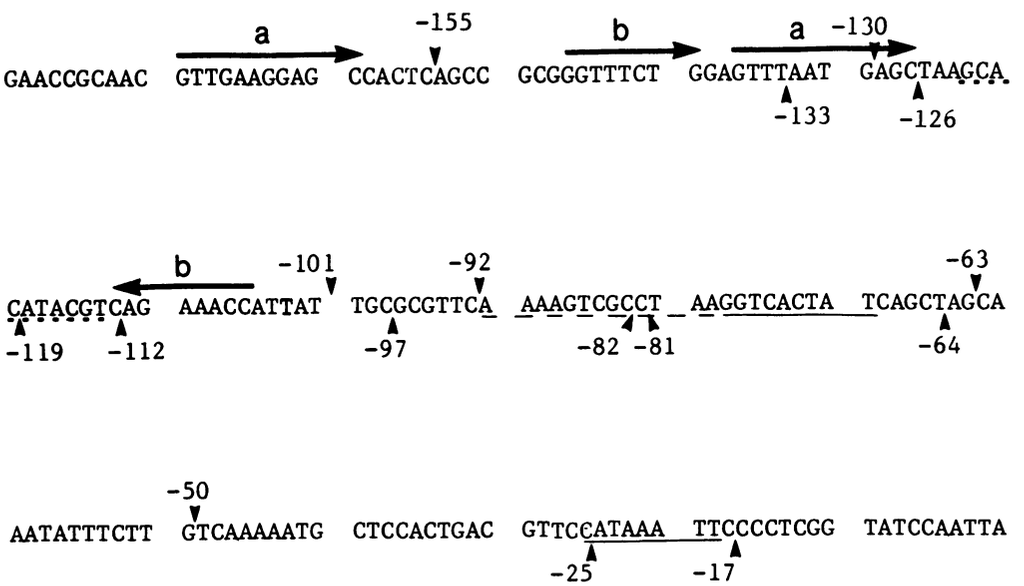

FIG. 1. *nos* promoter sequence showing deletion endpoints. Arrowheads above and below the sequence indicate 5′ and 3′ deletion endpoints, respectively. The arrows above the sequence represent an 11-bp direct repeat (*a*) and an 8-bp inverted repeat (*b*) (4). The dotted line below the sequence indicates a stretch of 10 alternating purine and pyrimidine residues that constitute a potential Z-DNA-forming region (Z). The CCAAT- and TATA-box homologous sequences are indicated by a solid line under the sequence, while the extended CCAAT-box region is indicated by a dashed line.

Table 1. Stable transformation analysis of the *nos* promoter mutants

Group	Deletion endpoint (3′/5′)	% of wild-type activity	Exp., no.
A	−64/−50	72.6	1
B	−64/−63	80.9 ± 0.4	4
	−81/−63	24.4 ± 5.5	3
	−82/−63	29.4 ± 6.0	4
	−97/−63	5.0 ± 1.0	4
	−112/−63	0	4
	−119/−63	0	5
	−126/−63	0	2
C	−25/−92*	59.0 ± 7.3	4
	−82/−92*	120.9 ± 22.5	2
	−97/−92	76.1 ± 12.2	4
	−112/−92	2.2 ± 0.6	5
	−119/−92	0	4
	−126/−92	0	3
	−133/−92	0	5
D	−81/−101*	101.7 ± 24.7	4
	−97/−101*	86.4 ± 16.4	6
	−112/−101	2.9 ± 1.7	5
	−119/−101	0	6
	−133/−101	0	1
E	−82/−130*	261.6 ± 29.2	3
	−97/−130*	364.5 ± 76.2	3
	−112/−130*	134.9 ± 34.6	2
	−119/−130*	88.4 ± 14.5	4
F	−81/−155*	269.0	1
	−97/−155*	316.6 ± 29.2	2
	−112/−155*	109.7 ± 32.0	3

*Duplication (all others are deletions).

activity, indicating that the functional CCAAT box element may extend beyond the originally recognized homology.

The small deletion −112/−101, which removed seven of the eight nucleotides of the downstream *b* element, resulted in a reduction of CATase activity by a factor of >30, which still left the CATase activity above background levels (Table 1, group D). This residual activity was totally eliminated in the deletion mutant −119/−101, which is missing an additional 7 bp. In this mutant, 6 of the 10 alternating purine and pyrimidine residues have been removed, destroying the Z-DNA-forming potential of the region. Thus, it appears that the core of the upstream activator may consist of these two unique features: a Z-DNA element and the *b* repeat.

To examine whether the upstream *a/b* repeat pair could functionally substitute for the downstream pair, the deletion mutant −133/−92 was constructed (Table 1, group C). In this construct, 42 bp of DNA, including the downstream *a/b* repeat and the Z-DNA, was removed and replaced by 9 bp of linker sequence, resulting in a net 33-bp deletion and putting the upstream *a/b* repeat pair in almost exactly the same position as the deleted downstream *a/b* repeat pair with respect to the CCAAT and TATA boxes. This construct resulted in a totally nonfunctional promoter, indicating that the differences between the upstream and downstream *a/b* repeat pairs are functionally significant. The most obvious explanation is that the orientation of the *b* element and/or the absence of a potential Z-DNA-forming region results in a nonfunctional upstream *a/b* repeat pair. Alternatively, the surrounding sequences may be important for promoter activity.

Functional Separation of the *nos* Promoter Elements. Two deletions between the TATA box and the CCAAT box region (−64/−50 and −64/−63) gave high levels of promoter activity—73% and 81% of wild type, respectively (Table 1, groups A and B). The deletion −97/−92 and the duplications −82/−92, −81/−101, and −97/−101 between the CCAAT box and the upstream element retain 76–121% of wild-type promoter activity (Table 1, groups C and D). The small effects may be due to alternation of spacing between the promoter elements or to deletion of a portion of the control elements.

Inversion of the Upstream Region. Many upstream activators such as enhancers function in both orientations (14, 32). To test this possibility, a 209-bp fragment of pGA355-97, which carries the *nos* promoter upstream region containing both *a/b* repeat pairs, was connected in either the correct or inverse orientation to the 5′ deletion mutants 553-92 and 553-101, neither of which were functional without the upstream element (4). The 209-bp fragment functioned only marginally in the inverted orientation (1.3–3.8%) compared to the wild-type orientation (76–86%). However, the level of CATase activity was consistently above background, indicating that an inverted orientation is partially functional (data not shown). Not only the orientation but also the spacing between the upstream element and the downstream promoter sequences were altered. Therefore, it leaves in question whether the reduced expression is due to orientation, spacing alterations, or interference by negative control sequences.

Duplication of Upstream Sequences. We also have studied the role of the essential upstream element by duplicating the upstream control regions. When the upstream region including both the Z-DNA and downstream *b* repeat was duplicated (−97/−130 and −82/−130), expression was tripled (Table 1, group E). However, when the region including only the potential Z-DNA-forming sequence was duplicated (−112/−130), expression was only moderately affected. Expression remained essentially at wild-type levels when the region immediately upstream of the "Z–*b*" essential element was duplicated (−119/−130). Duplication of a larger region (Table 1, group F) showed a similar pattern of activity. These results corroborate previous evidence that indicated that the Z-DNA and *b* element combined form the functional activator element.

Effect of Spacing on Promoter Activity. It was observed in the simian virus 40 early promoter that mutations that altered the spacing between promoter elements by a full turn (10.5 bp) of the DNA helix had a much less profound deleterious effect than those that altered the spacing by half of a turn of the helix (33). We examined the role of helical periodicity as well as spacing between each of the *nos* promoter elements on the activity of the promoter. A crude level of analysis revealed no correlation between promoter activity and deletion of an entire turn versus a partial turn of the helix (Table 2). A 66-bp duplication from −92 to −25 resulted in 59% of wild-type promoter activity, indicating that there is a great deal of latitude in the spacing requirements of the *nos* promoter.

Transient Expression Assay. Being a constitutive promoter expressed without developmental or environmental specificity, the *nos* promoter is well-suited for transient analysis. To test the fidelity of electroporation and transient analysis of promoter function, we compared the efficiency of various

Table 2. Effect of spacing between *nos* promoter elements

Elements	Deletion endpoint (3′/5′)	Spacing, bp	Helical turns	% of wild-type activity
CCAAT/TATA	−64/−50	−6	0.6	73
	−64/−63	+7	0.7	81
Upstream/CCAAT	−97/−92	+3	0.3	76
	−82/−92	+18	1.7	121
	−81/−101	+28	2.7	102
	−97/−101	+12	1.1	86
Upstream/TATA	−25/−92	+75	7.1	60

mutants at promoting gene expression under both stable (*Agrobacterium*-mediated) and transient (electroporation-mediated) conditions.

We performed initial tests to determine whether DNA conformation would affect the activity of the promoter elements. We found no significant difference in CATase activity for any of the promoter mutants depending on the state of the DNA (either linear or supercoiled), so we used supercoiled DNA throughout the remaining experiments.

In general, the results from the transient expression assays were different from the results of the stable expression assays (Table 3). Deletion of the TATA box (pGA515-25) resulted in a reduction in CAT activity by only a factor of 2 as compared to a reduction by a factor of ≈10 in the stable expression assay (4). Plasmids of the pGA553 (5′ deletion) series consistently gave low levels of expression compared to the pGA515 (3′ deletion) and pGA561 (internal deletion and duplication) plasmids. The pGA553 plasmids are different from the others in that they lack pBR sequences that may influence transient expression. When the pGA553 plasmids are considered independently, we see that deletion of the sequence between −205 and −130 causes expression to decrease by more than a factor of 2. Deletions to −101, −92, and −63 decreased CATase activity to background levels. These results are in agreement with the previous observations from stable transformation analysis (4).

Deletion of the small region between the TATA and CCAAT boxes (−64/−63) gave 67% in the transient assay and 81% of wild-type activity in the stable assay. Deletion of the CCAAT box homologous sequence (−81/−63) gave 50% activity in the transient assay and 24% activity in the stable assay. Further deletion of the CCAAT box region (−97/−63) reduced expression in both systems, although the levels were different: reduction to 15% in the transient system but to 5% in the stable system. Deletion of the region between the CCAAT box and upstream regions (−97/−92) gave 50% activity in the transient assay, similar to the values of CCAAT- or TATA-box deletions, as compared with 76% in the stable assay. Deletion of the downstream *b* repeat element (−112/−101) reduced expression to 45% in the transient assay as compared with 3% for the stable assays. Deletion of the upstream Z–*b* element (−119/−101) decreased CATase activity to background levels in both systems. It appears that crippled promoters show unusually high activity in the transient assay system, whereas removal of the entire upstream element results in no activity regardless of the assay. Duplications of the potential Z-DNA-forming sequence (−112/−130) increased expression moderately above wild-type levels, whereas duplication of the Z–*b* element region (−97/−130) tripled CATase activity, as observed in the stable transformation assay system. This further indicates that the upstream element acts in a similar fashion in both systems.

Table 3. Transient analysis of the *nos* promoter elements

Plasmid	Deletion endpoint (3′/5′)	% of wild-type activity	Exp., no.
pGA515	−17	100.0	
	−25	54.9 ± 6.2	5
pGA553	−205	35.3 ± 2.5	3
	−130	15.5 ± 2.0	3
	−101	0	2
	−92	0	3
	−63	0	3
pGA561	−64/−63	66.5 ± 13.1	3
	−81/−63	49.8 ± 8.3	6
	−97/−63	15.3 ± 1.9	3
	−97/−92	50.1 ± 12.9	3
	−112/−101	45.0 ± 8.8	6
	−119/−101	0	6
	−112/−130*	143.0 ± 22.4	3
	−97/−130*	329.8 ± 12.0	3

The pGA515 plasmids, which contain 3′ *nos* promoter deletions, and the pGA553 plasmids, which contain 5′ deletions, have been described (4). pGA561 plasmids contain internal promoter deletions and duplications as described in the text.
*Duplication (all others are deletions).

DISCUSSION

Analysis of the *nos* promoter by use of the promoter analysis vector (31) has given us a detailed description of the promoter. We have found that an essential upstream region is located between positions −130 and −97. A 12-bp deletion (−112 to −101) causes a reduction in promoter activity by a factor of 30, whereas a larger deletion (−119 to −101) reduces expression to background levels. This 19-bp deletion identifies a core region of the upstream essential element. These results are in contrast to the previous report that the CCAAT box sequence was the most distal functional element of the promoter (10). The differences are probably due to species or tissue specificity of the *nos* mutant promoters.

The core upstream essential element is contained within an interesting structural feature of the *nos* promoter region. We observed previously that two 11-bp (*a*) and two 8-bp (*b*) repeated elements exist in an alternating tandem array forming two *a*/*b* repeat pairs (4). The proximal portion of the core element overlaps the downstream *b* repeat, while the distal portion overlaps 10 nucleotides that will potentially form Z-DNA. A similar Z-DNA sequence was also found at the −180 region of the *ocs* gene (34). DNA sequences of the Z-DNA regions show significant homology (11 of 13 bp) between *nos* and *ocs* promoters. The *nos* Z-DNA is also highly homologous to the Z-DNA sequence within the consensus 15-bp region of zein genes at −330 where the trans-acting factor interacts (35). Such potential Z-DNA-forming sequences have been implicated in regulatory processes of other eukaryotic genes (36).

The upstream *a*/*b* repeat pair did not functionally substitute for the downstream *a*/*b* repeat pair. Two obvious differences are that the *b* elements are inverted complements of each other, and a potential Z-DNA-forming sequence does not exist between the *a* and *b* elements of the upstream repeat pair.

Duplication of the essential element tripled the strength of the *nos* promoter. However, inversion of the upstream region of the *nos* promoter results in only marginal promoter activity. Therefore, the *nos* upstream activator may be different from the upstream elements of *rbcS* (12–14), which is equally active in both orientations.

We have demonstrated that there are three blocks of essential regions, the TATA box, CCAAT box, and upstream elements. This is in direct contrast to what was observed with another T-DNA gene, the *780* gene, which showed no decrease in expression when the CCAAT box homologous sequence was deleted (37). Maintenance of precise spacing between the various promoter elements was not a strict requirement for *nos* promoter activity.

Transient analysis of the *nos* promoter gave results quite different from stable expression analysis. Transient analysis did not clearly reveal any structural features from the TATA box through the downstream *b* repeat. However, in both assay systems, deletion of the entire core upstream element abolished promoter activity, whereas duplication of the element enhanced the promoter strength. Although the method does not always reflect the results of the more natural stable integration analysis, this system may prove invaluable for the study of upstream elements and trans-acting factors.

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