Cis-acting regulatory elements controlling temporal and organ-specific activity of nopaline synthase promoter

Sam-Bong Ha and Gynheung An

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA

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ABSTRACT

Regulatory elements controlling temporal and organ-specific expression of the nopaline (nos) gene were identified by analyzing deletion mutants of the promoter. As observed in cultured cells, the TATA box element was required for efficient promoter function and the 29 bp upstream promoter region between -130 and -101 was necessary for the nos promoter activity in various vegetative organs. This 29 bp region includes ten nucleotides of a potential Z-DNA-forming sequence (Z element) and eight nucleotides of a repeated element (b element). Duplication of b elements significantly enhanced the promoter strength, revealing the importance of the element in all plant organs. Unlike the results in the cultured cells, however, deletion of the b element or CCAAT box region completely inactivated the promoter function in regenerated organs. Therefore, it appears that transcription initiation is more tightly controlled in differentiated plant cells than in cultured cells.

INTRODUCTION

Many eukaryotic genes are expressed in multiple cell types or at different developmental times (1). In order to understand the mechanism regulating expression of genes in different cell types, we have studied cis-regulatory elements controlling the nopaline synthase (nos) gene which is located on the transferred DNA (T-DNA) of Agrobacterium tumor-inducing (Ti) plasmid (2), and is efficiently expressed in a wide variety of plant tissues (3) when the gene is transferred into plant chromosomes (4,5). It was recently observed that the nos promoter activity is developmentally and tissue-specifically regulated in transgenic tobacco plants (6). The nos promoter is highly active in roots and lower parts of a plant but the activity gradually decreases toward the upper parts.

Studies of the nos promoter in cultured tobacco cells have identified at least three regulatory regions: 1) TATA box, 2) CCAAT box, and 3) upstream regulatory region (7,8). The upstream region is composed of two

distinct elements: an eight-bp repeated (b) element and a potential Z-DNA-forming sequence (Z) element (9). Deletion of the Z element abolishes the promoter activity, whereas deletion of other elements, such as the TATA box, CCAAT box, or b elements, reduces the promoter strength but does not diminish the promoter function in cultured tobacco cells (8,9).

MATERIALS AND METHODS

Strains and plasmids.

The E. coli strain MC1000 (10) was used as host for cloning experiment. Agrobacterium tumefaciens strain LBA4404, which carries a helper Ti plasmid pAL4404 (11), was used for trans-formation of tobacco plants. Nicotiana tabacum L. cv. Samsun and cv. Xanthi were maintained as sterile shoot culture. The nos promoter mutants have been described previously (7,8). Two chimeric molecules were constructed by fusing truncated chlorophyll a/b binding protein (cab) promoter and nos promoter. The 1.12 Kb fragment of Arabidopsis cab1 promoter which contains upstream sequence from CCAAT box was linked to the nos 5' deletion mutants, -155 and -101, respectively.

Plant transformation.

Plasmids were transferred to A. tumefaciens LBA4404 by direct DNA transformation (8,12). The structure of binary Ti plasmids in transformed Agrobacterium cells was verified by the modified alkaline lysis method (12). Tobacco leaves from sterile plants were sliced and stably transformed by the cocultivation method (13). Transformed calli were regenerated into whole plants, and at least six plants for each construct were individually analyzed for the chloramphenicol acetyltransferase (CAT) activity (12).

RESULTS

The nos promoter is highly active in roots and lower parts of a plant, but its activity gradually decreases in the upper parts (6). To identify cis element responsible for this vertical gradient of the promoter strength, mutant promoters containing different amounts of the nos 5' control region were fused to a reporter gene, cat. Effects of these mutations were analyzed in transgenic tobacco plants transformed using Ti-plasmid vector system. In order to minimize possible artifacts arising from surrounding chromosomal sequences, at least six independently

Fig. 1. Vertical gradient activity of 5' deletion mutant promoters. Relative promoter strength of six 5' deletion mutants was assayed in upper leaf (u), lower leaf (l), and root (r) of primarily transformed tobacco (*Nicotiana tabacum* L. cv. Samsun) by measuring conversion of ^{14}C -chloramphenicol (C) to acetylchloram-phenicol (AC). At least six independently transformed plants were analyzed for each mutant. Results from representative plants that exhibited an average level of CAT activity for each mutant are shown. The deletion end points of the 5' mutants (arrow), inverted repeat elements (●), potential Z-DNA-forming element (○), CCAAT box (striped box), and TATA box (open box) are indicated on the linear map of the *nos* promoter.

transformed tobacco plants were analyzed for the CAT activities driven from the mutant *nos* promoters.

Analysis of the 5' deletion mutants.

Six *nos* promoter mutants (-263, -167, -155, -130, -101, and -92) that carry different amount of the 5' control region were tested for promoter strength and specificity (Fig. 1). The promoter specificity was maintained when at least 130 bp of the control region was present. However, deletion of the 29 bp DNA fragment between -130 and -101 abolished the promoter activity. These results suggest that a regulatory element(s) located in this 29 bp fragment is necessary for the vertical gradient of the *nos* promoter activity. This region includes a ten bp stretch of alternating purine and pyrimidine nucleotides that is a potential Z-DNA-forming sequence (Z element) and an eight bp (b) element whose inverted repeat is located at the -140 region. These elements were previously identified as essential elements for *nos* promoter function in

Fig. 2. Effect of various mutations on the *nos* promoter activity in vegetative organs. The numbers shown on the right indicate relative promoter strength of 3' deletion (B), internal deletion (C), and duplication (D) mutants measured in upper leaf (u), middle leaf (m), lower leaf (l), total stem (S), and total root (R) of transformed tobacco plants as the % conversion of chloramphenicol to acetylchloramphenicol. Results from representative plants that exhibited an average level of CAT activity for each mutants are shown. Inverted repeat elements (●), potential Z-DNA-forming element (○), CCAAT box (striped box), TATA box (open box) and deleted DNA sequences (dots) are indicated on the linear map of the *nos* promoter.

cultured cells (8). The results in Fig. 1 also suggest that the sequence upstream from -130 is involved in enhancement of promoter activity in various vegetative organs as observed previously in cultured cells (7).

Analysis of 3' deletion mutants.

The effects of deletions in the TATA box region were examined

similarly (Fig. 2B). Deletion of the sequence downstream of the TATA box region (3'del -17) did not significantly alter the promoter activity, whereas further deletion into the TATA box (3'del -25) reduced the promoter strength by at least ten-fold without losing the promoter specificity. Measuring CAT activity may represent lower value of the promoter strength since mRNA initiated in the cat coding region would produce non-functional protein. However, it is more likely that the transcription initiation occurred upstream of the cat coding region, since there is 45 bp DNA sequence between the deletion end point and the ATG initiation codon. The results from the 5' and 3' deletion mutants indicate that the DNA sequence between -130 and the TATA box region is essential for the promoter function and that the surrounding sequences enhance the promoter activity.

Analysis of internal deletion mutants.

In order to study the role of elements located between -130 and the TATA box, internal deletion mutants were examined for the promoter activity in plants (Fig. 2C). Deletions of the CCAAT box region (int. del. -81/-63 and -97/-63) abolished the promoter activity in all plant organs, including root. This result is different from that obtained from cultured cells. Deletion of the CCAAT box region reduced the promoter strength by five- to twenty-fold, but did not abolish the promoter activity in cultured tobacco cells when it was analyzed by either stable or transient assay (8).

Deletion of 5 bp between the CCAAT box and upstream region (-97/-92) did not affect promoter activity. The result is consistent with the previous finding in cultured cells (8). However, deletions of the upstream regulatory region (-112/-101, -119/-101, and -133/-92) completely abolished promoter activity in all organs. It was previously observed that the Z and b elements located in this region are important for the nos promoter function in cultured tobacco cells (8). However, deletion of the b element alone reduced, but did not abolish the promoter activity in transformed calli. The results in this study indicate that the b element located downstream of the Z element is essential for the nos promoter function in differentiated organs.

Analysis of duplication mutants.

To further examine the role of the upstream elements located between -130 and -101, duplication mutants of this upstream region were tested for CAT activity in plants (Fig. 2D). Duplication of the 32 nucleotides carrying both Z and b elements significantly enhanced the

Fig. 3. Cab1-nos hybrid promoter activity. Organ-specific expression of chimeric promoters between the 1.12 Kb fragment of *Arabidopsis cab1* promoter upstream region and the *nos* mutant -101 (A) or -155 (B). The *cab1* promoter carrying the 1.40 Kb intact *cab1* promoter (A) and the *nos* promoter -155 (B) carrying all of the necessary elements for promoter function in vegetative organs were used as controls. The CAT activities were measured from upper leaf (u), middle leaf (m), lower leaf (l), and total root (R).

promoter activity (dup -97/-130) by one order of magnitude. Such an effect was not observed by duplicating the Σ element alone. These results, along with deletion mutant analyses, strongly suggest that this immediate upstream region carrying both elements controls the promoter function in all vegetative organs.

Analysis of cab1-nos hybrid promoters.

We have obtained further evidence that this upstream region is involved in the gradient activity of the *nos* promoter by testing hybrids of *nos* and *Arabidopsis cab1* promoters (Fig. 3). When the defective *nos* promoter -101 (Fig 1, 5' mutant -101) was fused to a truncated *cab1*

promoter which carries the upstream regulatory elements for the organ-specific expression of the photosynthetic gene, but which is non-functional by itself due to lack of the proximal region, the characteristics of the hybrid promoter were the same as that of the intact cab1 promoter, i.e. the promoter was almost equally active in leaves regardless of position but the promoter activity was weak in roots (Fig. 3A). However, another hybrid promoter between the nos -155 (Fig. 1) and the cab1 upstream region was active in all vegetative organs tested, including roots (Fig. 3B) and showed additive characteristics of both promoters. The results of these chimeric promoter studies also suggest that the immediate upstream region of the nos promoter is primarily responsible for the gradient expression. These observations are in conflict with the findings of Simpson et al. (14), who showed that the upstream region of the pea cab gene suppressed activity of the nos promoter in root. This discrepancy might be due to the differences in the promoter organization of different cab genes.

DISCUSSION

T-DNA genes are normally silent in its natural host, Agrobacterium, but they become active in a wide range of plant cells when the genes are stably integrated into nuclear chromosomes (3,4,5). The nos gene produces one of the most abundant transcripts in crown gall tumors incited by the nopaline type Ti-plasmids (5). Therefore, the nos promoter has been frequently used for expression of foreign genes in plants (6,15) or for the construction of chimeric selectable markers, such as a fusion between the nos promoter and the neomycin phospho-transferase (npt) gene (16,17,18). It has been described in several previous publications that the nos promoter is constitutively active in plants (14,19,20,21). However, it was recently observed that the nos promoter activity is developmentally and organ-specifically regulated in plants (6). In order to further understand the molecular mechanisms controlling the specificity of the promoter, we have examined deletion mutants that lack a portion of the nos 5' control region in transgenic tobacco plants. The results indicate that the TATA box region and the sequence between -155 and -130 are required for maximum promoter activity and that sequence between the TATA box and -130 are necessary and sufficient for the specific activity of the nos promoter. It appears that the immediate upstream region between -130 and -101 retains the regulatory elements

controlling temporal and organ-specific expression of the nos promoter. Duplication of both Z and b elements enhanced the promoter activity in all plant organs tested, suggesting that both elements are involved in control of the nos promoter activity. Unlike previous results obtained from cultured cells, deletion of the b element or the CCAAT box region abolished the promoter activity in regenerated tobacco plants. Therefore, it appears that the nos promoter is more tightly regulated in plant organs than in cultured cells. The leaky expression from the nos promoter in undifferentiated cells is probably due to lack of an active negative factor or to an altered specificity of trans-acting regulatory factors and the promoter sequence.

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REFERENCES

1. Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) *Science* 236, 1237-1244.
2. Willmitzer, L., Dhaese, P., Schreier, P.H., Schmalenbach, W., Van Montagu, M. and Schell, J. (1983) *Cell* 32, 1045-1056.
3. Simpson, J., Van Montagu, M. and Herrera-Estrella, L. (1986) *Science* 233, 34-38.
4. Chilton, M.-D., Drummond, M.H., Melro, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. and Nester, E.W. (1977) *Cell* 11, 263-271.
5. Joos, H., Inze, D., Caplan, A., Sormann, M., Van Montagu, M. and Schell, J. (1983) *Cell* 32, 1057-1067.
6. An, G., Costa, M.A., Mitra, A., Ha, S.-B. and Marton, L. (1988) *Plant Physiol.* 88, 547-552.
7. An, G., Ebert, P.R., Yi, B.-Y. and Choi, C.-H. (1986) *Mol. Gen. Genet.* 203, 245-250.
8. Ebert, P.R., Ha, S.B. and An, G. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5745-5749.
9. Mitra, A. and An, G. (1988) *Mol. Gen. Genet.*, in press.
10. Casadaban, M.J. and Cohen, S.N. (1980) *J. Mol. Biol.* 138, 179-208.
11. Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) *Nature* 303, 179-181.
12. An, G. (1987) *Methods Enzymol.* 153, 292-305.
13. An, G., Watson, B.D. and Chiang, C.C. (1986) *Plant Physiol.* 81, 301-305.

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14. Simpson, J., Schell, J., Van Montagu, M. and Herrera-Estrella, L. (1986) *Nature* 323, 551-554.
 15. Schell, J.S. (1987) *Science* 237, 1176-1183.
 16. Bevan, M.W., Flavell, R.B. and Chilton, M.D. (1983) *Nature* 304, 184-187.
 17. Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg S.B., Hoffmann, N.L. and Woo, S.C. (1983) *Proc. Natl. Acad. Sci. USA* 80, 4403-4807.
 18. Herrera-Estrella, L., Depicker, A., Van Montagu, M. and Schell, J. (1983) *Nature* 303, 209-213.
 19. Herrera-Estrella, L., Van Den Broeck, G., Maenhaut, R., Van Montagu, M., Schell, J., Timko, M. and Cashmore, A. (1984) *Nature* 310, 115-120.
 20. Simpson, J., Timko, M.P., Cashmore, A.R., Van Den Broeck, G., Schell, J., Van Montagu, M. and Herrera-Estrella, L. (1985) *EMBO J.* 4, 2723-2729.
 21. Timko, M., Kausch, A.P., Castresana, C., Fassler, J., Herrera-Estrella, L., Van Den Broeck, G., Van Montagu, M., Schell, J. and Cashmore, A.R. (1985) *Nature* 318, 579-582.