Volume 12 Number 20 1984 Nucleic Acids Research

A functional map of the nopaline synthase promoter

C.H.Shaw, G.H.Carter, M.D.Watson and C.H.Shaw*

Department of Botany, University of Durham, Science Laboratories, South Road, Durham DH1 3LE, UK

Received 13 August 1984; Revised and Accepted 25 September 1984

ABSTRACT

This paper describes the first functional map of a promoter expressed from the plant chromosome. We have constructed a series of overlapping deletion mutants within the region upstream of the Ti-plasmid encoded nopaline synthase (nos) gene. By monitoring nos expression in tumour tissue we have inferred a functional map of the nos promoter. The maximum length of sequence upstream of the transcription initiation point required to express wild type levels of nopaline synthase is 88 bp. Within this region, the "CAAT" box is essential for maximal activity; deletion of this sequence reduced apparent nos expression by over 80%. Presence of an intact or partial "TATA" box in the absence of the "CAAT" box supports a barely detectable level of nopaline synthase. Removal of all sequences upstream of the nos coding sequence results in no detectable activity.

INTRODUCTION

The genes encoding several plant proteins have recently been isolated and sequenced[1-8]. Putative regulatory signals within the 5' flanking sequences have been identified by analogy to animal genes, where functional analysis has demonstrated the significance of conserved sequence blocks[9-17]. Thus sequences resembling "CAAT" (GG(C/T)CAATCT) or "TATA" (TATA(A/T)A(T/A)) boxes are present in the upstream sequences of many plant genes[18]. However, apart from the observation that transcription usually initiates at a position some 30 bp downstream of the "TATA" box[4,6,7,19,20] there is as yet no direct demonstration that conserved sequence blocks have a role to play in the expression of plant genes. The major impediment has been the lack of a system with which to perform functional analysis of plant promoters.

The Ti-plasmids of Agrobacterium tumefaciens can provide both the mechanism for DNA transfer, and the necessary sequences to promote expression of foreign genes[21-24]. Following transfer of the T-DNA to the plant cell, several genes encoded by this element are expressed, of which the most heavily transcribed are those involved in opine synthesis e.g. nopaline synthase[25,26]. The region encompassing the nopaline synthase (nos) gene

depicted in Fig. 1, has been completely sequenced, and the transcription initiation site pinpointed[27,28]. Furthermore, the nos promoter has been used to promote the transcription in plant cells of bacterial antibiotic resistance genes[29-32]. However aside from the demonstration that sequences beyond 261 bp upstream of the mRNA cap site are not required for nos expression[33] no functional analysis of the nos promoter has been performed. Despite being present on a plasmid in a prokaryote, the nos gene is expressed from the plant nuclear genome, and is functionally therefore eukaryotic. This is borne out by the presence of conserved sequence blocks in the nos upstream region, resembling "CAAT" and "TATA" boxes[27,28] as would be expected of a promoter transcribed by RNA polymerase II[34].

The Ti-plasmid mediated gene transfer systems developed during the last few years, also allow the easy mutagenesis of T-DNA genes, and their introduction into plants[23,35-41]. Thus, we have embarked upon a project to functionally map the region upstream of the nos mRNA cap site (Fig. 1) using a collection of deletion mutants generated by Bal 31 exonuclease. As the right border of the T-region is only some 300 bp upstream of the cap site, the phenotypes of the deletions fall into two classes, affecting either tumour formation, or nos expression. We have reported elsewhere that deletion of the right hand copy of the 25 bp repeats which flank the T-region almost completely abolishes tumour formation[42]. Here we report the properties of the second class of deletion mutants, those influencing nos expression. Using these mutants we have inferred a functional map of the nos promoter, the first such analysis performed upon a gene expressed from the plant chromosome.

MATERIALS AND METHODS

Microbiological Techniques

Bacterial growth conditions, antibiotic concentrations, and DNA purification were as previously described[40].

General cloning procedures

Conditions for restriction enzyme digestion, ligations, DNA fragment isolation and manipulation of EcoRI linkers, were as previously described[40]. Bal 31 digestion was performed at 37°C for 30 seconds, using low salt (0.2 M NaCl) conditions[43] and terminated by the addition of phenol.

Deletion construction

Deletions were constructed (Fig. 2) in pASK1029[40] a pBR322 based replicon containing HindIII fragment 23, a 3.2 Kb fragment spanning the right border of the nopaline Ti-plasmid pTiC58 T-region. The pBR322 moiety of pASK1029 lacks both EcoRI and Bam HI sites. pASK1029 was cleaved at the unique SstII

site and the resulting cohesive termini resected for 30 seconds with Bal 31. After ligation of EcoRI linkers, and digestion with EcoRI, a 1.2 Kb EcoRI fragment, originally derived from Tn903, expressing kanamycin/neomycin resistance was ligated in place. Kanamycin resistant transformants of E.coli were selected, and screened by "mini-lysate"[44]. Plasmids purified from promising candidates, renamed pDUB1106Δ ....., and mapped by restriction enzyme digestion and gel electrophoresis. pDUB1106Δ .... derivatives were then digested with PstI, ligated to similarly digested pGV1106[45] and introduced into competent E.coli cells by transformation, selecting for resistance to gentamycin and neomycin. These plasmids were renamed pDUB1201Δ ....

Introduction of deletion mutants into the pTiC58 derivative pGV3105

pDUB1201Δ .... derivatives were transmitted to A. tumefaciens C58C'Rif^R (pGV3105) by pRN3 as previously described[35,40], with selection for transconjugants resistant to rifampicin and neomycin. Following a Ti-plasmid mediated conjugation to A. tumefaciens C58C'Ery^{R}Cm^R, transconjugants selected for resistance to neomycin, erythromycin and chloramphenicol were screened for sensitivity to gentamycin, and such isolates renamed, eg pDUB1003Δ31 etc These were then purified and the correct insertion of the deletions was checked by Southern blotting of total A. tumefaciens DNA using HindIII-23, or the Tn903 neomycin fragment as probe[46].

Tumour induction

Overnight cultures of A. tumefaciens C58C'Ery^{R}Cm^R (pDUB1003Δ ....) were inoculated onto leaves or stems of Kalanchöe diaigremontiana using a sterile syringe needle. All plants were also inoculated with A. tumefaciens C58C'Ery^{R}Cm^R, and A. tumefaciens C58C'Ery^{R}Cm^R (pGV3105) as controls. Infected plants were maintained under fluorescent lighting, utilising a 16 hour on/8 hour off cycle at 25°C.

Nopaline synthase assays

Nopaline was detected in tumour tissue using a modification of published protocols[47,48]. Stem or leaf tumours were cut from infected plants, and a weighed amount (usually 50-100 mg) of tissue incubated for 16 hr at 28°C in 0.5 ml of Linsmaier and Skoog medium containing 0.1M arginine. The tissue was then removed, blotted dry, homogenised and pelleted in a microcentrifuge. 20 µl of the supernatant (usually 20-50 µl) was spotted onto the anodal side of a sheet of Whatman 3MM paper, and electrophoresed for approx 60 minutes, using as running buffer 5% formic acid, 15% acetic acid, in H_2O, pH 1.8. After drying in warm air the paper was sprayed with a 1:1 mixture of 0.2% phenanthrenequinone in ethanol, and 10% NaOH in 60% ethanol, and finally dried in cold air. After visualisation under long wave U.V. light, comparison

of test spots with standard spots containing 10, 5, 1 or 0.5 µg nopaline run on the same electropherogram allowed an estimate of the amount of nopaline present to be made. This was then converted to µg nopaline/g tissue. Nopaline levels synthesised in tumours induced by the various mutants were then compared to, and expressed as a proportion of, the amounts of nopaline synthesised in identically aged tumours incited on the same plant by the wild type parent A. tumefaciens C58C'Ery^{R}Cm^R (pGV3105) using tissue infected with an isogenic Ti-cured strain, A. tumefaciens C58C'Ery^{R}Cm^R as a negative control. Independent stem tumours, of identical age, induced upon separate plants were found to synthesise 280 $\pm$ 30 µg nopaline/g tumour tissue under these assay conditions, well within the published range[49].

DNA sequencing

All subcloning and DNA sequencing procedures were as recommended in the M13 cloning and sequencing handbook produced by Amersham International. To define the deletion endpoints, in the downstream direction, approximately 1 Kb EcoRI-Bam HI fragments were subcloned from each pDUB1106Δ... derivative into EcoRI-Bam HI cleaved M13mp9[50]. These fragments represent the region beginning at the new EcoRI site inserted into the nos upstream sequence, and extending to the Bam HI site situated approximately 850 bp downstream of the transcription initiation point. DNA sequencing was performed using dideoxynucleotide chain termination techniques[51] with α^{35}S-dATP as radiolabel, upon templates purified from single plaque isolates.

RESULTS

Construction of mutants

The strategy utilised for deletion construction (see Fig. 2 and Materials and Methods) is a derivation of that described for the introduction of foreign DNA, and thus site specific mutations into the nopaline plasmid pTi-C58[35,40]. The complete nos gene is contained within HindIII fragment 23 which spans the right T-region border (Fig. 1) and is present on the pBR322-based replicon pASK1029[40]. Transcription initiates at a point approximately 300 bp from, and proceeds away from, the right T-region border, marked by the 25 bp repeated element. The conserved sequence blocks, "TATA" and "CAAT" begin at nucleotide positions -26 and -78 numbering from the mRNA cap site. Beyond these is positioned a single SstII site at -150, with the normal right border being at -304. Thus deletions were constructed in pASK1029 by Bal 31 resection at the unique SstII site (Fig. 2). Potentially interesting deletions were identified by a combination of mini-lysate, restriction endonuclease mapping and DNA sequencing. Using the incW plasmid pGV1106[45] to provide functions

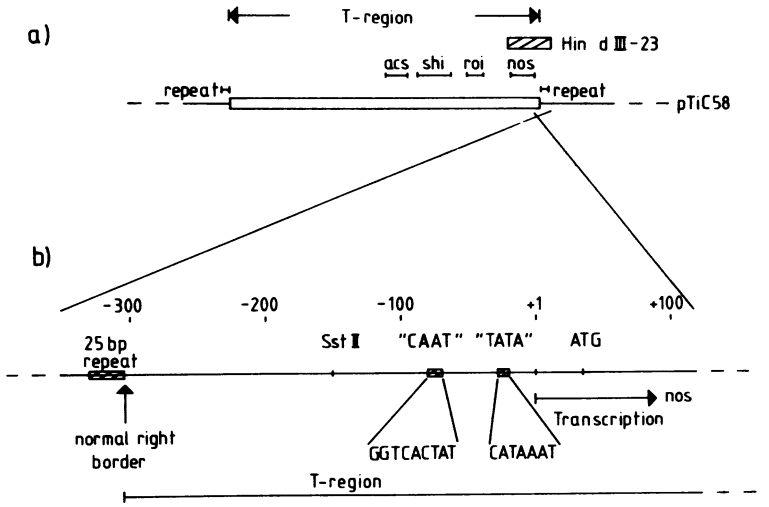


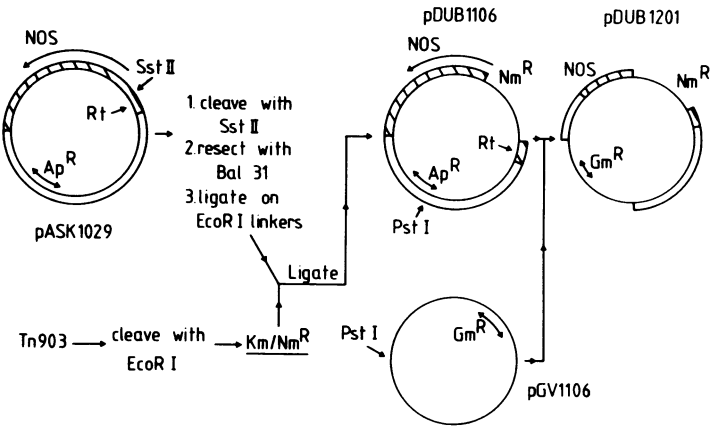
Fig. 1 a) Schematic diagram of the T-region of the nopaline plasmid pTiC58. The T-region is depicted as an open box, flanked by 25 bp direct repeats, the hatched box representing the area contained within HindIII fragment 23. Genetic loci represented are involved in agrocinopine synthesis (acs) shoot inhibition (shi) root inhibition (roi) and nopaline synthesis (nos).

b) Enlarged, and reversed (relative to Fig. 1a) section spanning the right border and nos promoter of the T-region. Numbers (in bp) refer to positions relative to the nos transcription initiation point, indicated by +1. The upstream regions which share DNA sequence conservation with "CAAT" and "TATA" boxes are shown enlarged beneath the map. ATG indicates the first methionine codon of the nos coding region.

necessary for replication in Agrobacterium, the mutated copies of HindIII-23 were inserted into the nopaline Ti-plasmid pGV3105 (pTiC58) to replace the wild type nos gene, using published protocols[35,40,42]. The deleted Ti-plasmids thus constructed (pDUB1003Δ...) were checked by Southern blotting and their oncogenic properties ascertained by inoculation onto wounded leaves or stems of Kalanchoe diaigremontiana.

Properties of constructed mutants

The characteristics of the non-oncogenic mutants constructed using this technique have been described elsewhere[42]. The mutations described in this paper have no significant effect on oncogenicity[42]. However when the nopaline synthase activity induced in these tumours was compared to wild type tumours (see Materials and Methods) it was found that the effect of removing increasing lengths of DNA sequence from the nos upstream region was a stepwise reduction in enzyme activity, consistent over several replicates. The extent



Construction of NOS promoter deletions

Fig. 2 Construction of nos promoter deletions. See text for details. The hatched box represents HindIII fragment 23 of pTiC58, the open box pBR322. Rt, right border; Abbreviations refer to genes encoding resistance to ampicillin (Ap^R) kanamycin (Km^R) neomycin (Nm^R) and gentamycin (Gm^R),

of the constructed deletions as determined either by restriction enzyme mapping or DNA sequencing is depicted diagrammatically in Fig. 3. Alongside this are shown the levels of nopaline synthesised in the tumours incited by the various mutants, under assay conditions, relative to wild type amounts. Fig. 4 illustrates the deletion endpoints within the nos upstream sequence as determined by DNA sequencing.

The method employed for deletion construction, results in mutants from which varying lengths of DNA have been removed from either side of the SstI site at -150. Therefore, the deletion of sequences upstream or downstream of this position could theoretically influence nos expression. However, the results demonstrate that removal of sequences between -302 and -88 does not diminish enzyme activity. On the contrary mutant Δ31, whose deletion extends from -302, 2 bp internal of the right border[42] to -130, and mutant Δ71, with a deletion extending from approximately 30 bp upstream of the SstII site to -122, both synthesize slightly greater than wild type amounts of nopaline under assay conditions. Mutant Δ77 has a deletion extending from approximately 80 bp upstream of the SstII site to -88, 10 bp upstream of the CAAT box. Yet, the nopaline synthase activity induced by this mutant is similar to that induced by the wild type parent pGV3105. It

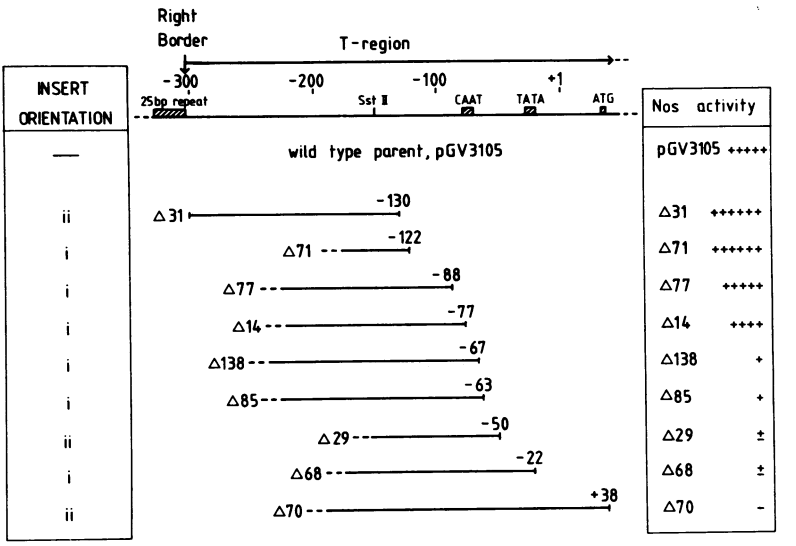


Fig. 3 Characteristics of deletion mutants. In the central panel, the region illustrated, its orientation and numbering are the same as for Fig 1b. Beneath this map are shown the boundaries of the deletion in the various mutants, depicted by a horizontal line, with the mutant numbers to the left of it. Where deletion endpoints were determined by DNA sequencing, the lines terminate in a vertical bar, with a number above it, and with a dotted edge for endpoints, estimated by restriction enzyme mapping. The panel to the left indicates the orientation of the kanamycin/neomycin resistance insert present within each deletion. The panel to the right presents the nopaline synthase activity for each mutant. Nopaline levels synthesised in tumours induced by the various mutants under assay conditions were calculated as described in Experimental Procedures. These values were then expressed as a proportion of the nopaline synthesised and detected using the same technique in identically aged tumours, induced by the wild type parent, pGV3105 on the same plant. The results illustrated represent average values determined from a minimum of six separate assays on 20–40 day old tumours, from several different inoculation sites. +++++ = 100% of wild type activity; - = no detectable activity, ± = <5% of wild type activity.

is thus pertinent to consider the effect of removal of progressively longer regions of DNA extending in the downstream direction from the SstII site towards the transcription initiation point.

Sequences upstream of -88 appear to be non essential for nos expression, and their removal may actually enhance it. Deletion of sequences between -88 and -77, including the first base of the CAAT box results in a slight fall in the level of activity detected (mutant Δ14). However, progressively longer deletions towards the transcription initiation point produce significant reductions in nopaline synthase activity. Thus, removal of the next 10 bp, including the complete "CAAT" box, reduces nos activity to below 20% of wild

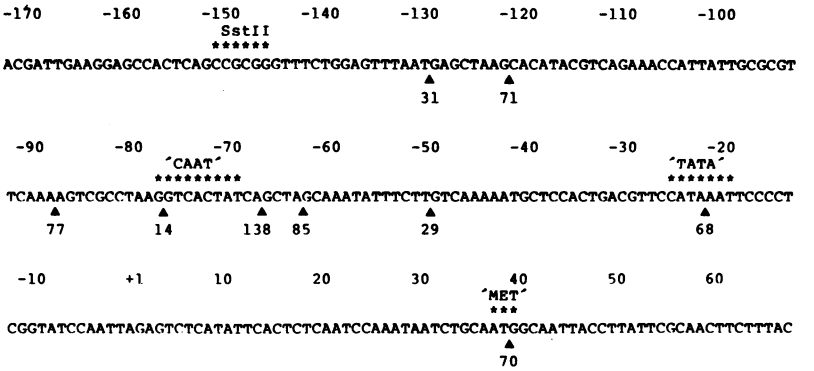


Fig. 4 DNA sequence analysis of deletion endpoints. The sequence depicted, derived from and numbered according to Depicker et al., (1982), is presented in the same orientation as Fig. 1b, and represents the nos sense strand of the closely related nopaline Ti-plasmid pTiT37 from immediately upstream of the SstII site, to beyond the first nos methionine codon. The genotypes of the various mutants are indicated below this by a solid triangle, above the mutant number, the point of the triangle indicating the sequenced endpoint of the deletion carried by that mutant. Asterisks denote the "CAAT", and "TATA" boxes, the initiator codon (MET) and the SstII site.

type (mutant Δ138). Although this level does not appear to be significantly reduced by a further 4 bp deletion (mutant Δ85) mutant Δ29, with its endpoint at -50, 13 bp beyond Δ85, supports only a much lower level of activity. This is reduced still further to barely detectable amounts in tumours incited by mutant Δ68, whose deletion terminates at -22, removing all but the last 3 bases of the "TATA" box. Thus, it was only in the minority of cases that any nopaline synthase was detected in mutant Δ68 tumour tissue. Mutant Δ70 which has its endpoint within the first methionine codon of the nos gene, failed to produce detectable levels of nopaline synthase under any circumstances.

DISCUSSION

Transfer of foreign genes to plant cells using the Ti-plasmids, and their expression promoted by nos upstream sequences is now an established fact[29-32]. Detecting such expression poses problems in many cases. Efficient purification of mRNA from plant cells is notoriously difficult, and furthermore this is complicated by the fact that the T-DNA transcripts together comprise less than 0.001% of the polyA$^+$ RNA population of tumour cells[26,52]. Moreover several months must usually elapse following transfer, before sufficient amounts of axenic tumour tissue are available for transcript analysis. These facts taken together make estimation of promoter activity in plant cells via

analysis of transcript levels a slow, difficult and laborious task. However, enzymatic assays for nopaline synthase activity are sensitive, semi-quantitative, require milligram amounts of tissue and may be performed within days of transfer[47-49]. For these reasons we thus chose to use nopaline synthase assays as an indicator of nos expression.

The use of an enzymatic assay as an indicator of gene activity is not entirely without precedent. Thus following introduction into plant cells of chimaeric genes, consisting of the nos promoter fused to coding sequences derived from bacterial antibiotic resistance genes, the presence of the genes was inferred from a positive enzymatic assay[29,30]. Furthermore Koncz et al[33] utilised the assay described here in order to determine the minimum lengths of the nopaline and octopine synthase promoters. Moreover, this same assay was used quantitatively to distinguish progeny of tobacco crown gall regenerants which were homozygous for the T-DNA from hemizygotes[53]. Thus this assay has already been used to provide an indication of the extent of gene expression.

Previous workers have demonstrated a correlation between the amount of nos transcription and the levels of nopaline synthase activity in tumour tissue[26,54]. Therefore, making the reasonable assumption that the influence of translational control, or enzyme turnover, will affect nopaline synthase levels induced in the different tumours by the mutants described here and their wild type parent to the same proportionate extent, the amounts of enzyme detected in this assay should mirror the extent of transcription of the nos gene. Thus the results presented here can be used to provide an approximate estimation of the residual promoter strengths of the mutants.

The effect of removing progressively longer stretches of DNA from the nos upstream region is to reduce the level of nopaline synthase activity detected. As previous experiments have demonstrated that the region extending from -261 to immediately downstream of the nos mRNA cap site contains all sequences necessary to promote nos transcription[29-33], we believe that the observed phenotypes of our mutants is due to the deletion of sections of the nos promoter. However it is important to consider alternative explanations for our results. One possibility is that the observed reduction in nos activity is due to an effect on the oncogenic properties of the mutants, either via changes in the proportion of transformed cells in the tumours, or the copy number of the T-DNA. We have previously demonstrated that deletion of the right copy of the 25 bp repeats flanking the T-region abolishes tumour formation, apparently due to an effect on T-DNA transfer[42]. However sequences

mapping from 2 bp internal of this repeat copy at -302, to within the nos coding sequence appeared to be unnecessary for the oncogenic process[42]. Thus the mutants described in this paper, which have deletions spanning from -302 (mutant Δ31) to +38 (mutant Δ70) show no significant differences from the wild type parent pGV3105 in their ability to form tumours[42]. Therefore, it is unlikely that changes in oncogenic properties can explain the observed reduction in nos activity. One factor worthy of mention is the presence of low numbers of the inciting bacteria within the tumour during the assay period. However, as the deletions affect neither their oncogenic ability nor their nopaline catabolic functions, the effect of this presence will be minor and consistent throughout the assay for all the mutants and the wild type parent. A further possible explanation for the observed phenotypes could be that the deletions cause indirect effects on the nos promoter, for instance via an influence upon DNA methylation. A reduction in the extent of methylation of the nos upstream region in Flax tumours has been correlated with an increase in nos expression[54]. However it is difficult to see how deletion of these sequences could result in a fall in nos activity, merely due to an effect on methylation. Furthermore this does not appear to be a universal phenomenon, as little or no T-DNA methylation was reported in nopaline crown galls of tobacco[55].

As all the mutants constructed are actually deletion insertion mutants, it is relevant to address the question as to whether the insert itself has any effect on the phenotypes observed, either directly or indirectly. It would appear that neither the mere presence of the insert, nor the separation it produces of sequences either side of the SstII site is inhibitory to nos expression (cf mutants Δ31, Δ71 and Δ77). Furthermore it is unlikely that the insert itself provides sequences capable of functioning as a promoter in combination with the remnants of the nos sequence, as it does not appear to be able to rescue a promoter-deleted gene nor complement a partially deleted one. Thus the effect of the insert would appear to be minimal.

A functional map of the nos promoter

The mutants described here display stepwise and consistent reductions in nos activity which correlates with the extent of their deletions. Although alternative explanations are possible, the most likely explanation of the observed phenotypes is that the deletions remove sections of nos upstream sequence essential for gene expression. Thus the following tentative conclusions can be made regarding the section of DNA between the right border of the T-region and the nos coding sequence:

1. The maximum length of DNA sequence upstream of the mRNA cap site required

for the expression of wild type levels of nopaline synthase in Kalanchöe diaigremontiana is 88 bp. The corollary of this is that sequences upstream of -88 are apparently non-essential for full nos expression.

2. The "CAAT" box (nos sequence GGTCACTAT) is essential for maximal nos activity. Deletion of this sequence reduces the apparent expression by over 80%.

3. A partial or complete "TATA" box (nos sequence CATAAAT) in the absence of the "CAAT" box supports a very low level of nos activity.

4. Removal of the "CAAT" and "TATA" boxes, the mRNA cap site, and the first two bases of the initiator codon, reduces nos expression to below detectable levels.

Previous studies using this assay have demonstrated that sequences upstream of -261 are non essential for nos expression[33]. The results presented in this paper thus further refine this analysis. On the basis of tumour phenotypes, two groups have recently reported the localisation of the promoters of two T-DNA genes[56,57]. These analyses have demonstrated that sequences beyond 72 bp and 121 bp upstream of the transcription initiation points of the transcript 2 and tmr genes respectively are non essential for the production of the wild type phenotype. However, there is as yet no published information regarding the level of expression necessary to maintain the wild type phenotype of these genes. Thus this paper presents the first functional analysis of a chromosomally expressed plant promoter. Therefore, the only points of comparison for these results, are other eukaryotic genes particularly those derived fron animals.

These results are similar in many respects to those produced during studies performed on animal promoters, where with some exceptions, complete expression in vivo requires functional "CAAT" and "TATA" boxes[15,16,58,59]. However based on upstream sequence conservation between several genes, Messing et al[18] have proposed that plant genes possess an "AGGA" and not necessarily a "CAAT" box. It is important to note therefore, that the conserved blocks "CAAT" and "TATA" within the 88 bp upstream of the nos transcription initiation site more closely resemble the animal consensus sequences than those proposed by Messing et al[18]. However, the nos promoter, like the other T-DNA promoters, is capable of being expressed in a wide variety of plant cells, e.g. tobacco[47] Arabidopsis[48] and Kalanchoe. Thus, it might be expected that such a general purpose promoter would be somewhat different from tissue- or species-specific promoters. The fact that it is expressed in such a variety of tissues and plants, implies that it satisfies the requirements

of RNA polymerase II in such varied environments. It is therefore relevant to discuss out tentative conclusions in the light of previous experiments.

The demonstration in this paper that sequences upstream of -88 are apparently non-essential for complete nos expression is not wholly unexpected. Indeed such a phenomenon has been observed with some animal genes[60]. However many animal genes do require sequences beyond 100 bp upstream of the mRNA cap site, e.g. histones[61] and for β-globin, expression of the gene in non-erythroid cells requires the presence not only of functional "CAAT" and "TATA" boxes, but also the SV40 enhancer[62]. Moreover, expression of the T-DNA encoded ocs gene requires sequences between -170 and -294[33], when assessed using the assay employed here, although it is worth pointing out in this context that the ocs upstream region lacks a sequence homologous to "CAAT", and contains two potential "AGGA" boxes[63]. It is therefore entirely possible that the two promoters have evolved in significantly different manners, and thus have distinct sequences involved in gene expression. We are currently initiating experiments to clarify this distinction.

Experiments using mutants whose deletions extend from the SstII site, towards and across the right border, have demonstrated that sequences up to -302, reading from the nos mRNA cap site, are not required for tumour formation[42]. However, the copy of the 25 bp repeat, situated immediately beyond this position, is essential for this process[42]. The results of our deletion analysis thus indicate that sequences between -88 and -302, upstream of the nos mRNA cap site are not required either for tumour formation or maximal nos expression. Our results do suggest that they may play a minor role in the regulation of nos expression. However, as the data appertains only to Kalanchöe leaves and stems, it is possible that the function of sequences between -88 and -302 in the nos upstream region, be it stimulatory or inhibitory, is more evident in other plants or tissues.

Complete deletion of the "CAAT" box results in over 80% reduction in nos activity. Our results also suggest that this box must remain intact for maximal expression, although the resolution of our analysis does not permit an unequivocal interpretation. These results are however in keeping with studies on animal genes. For example, deletion of the first two G residues from the rabbit β-globin "CAAT" box (GGCCAATCT) which bears a perfect homology to the consensus sequence, reduces transcription efficiency by almost 90%[16]. This underlines the importance of the nos "CAAT" box for gene expression.

In other systems, the "TATA" box has been identified as a sequence which

defines the specificity of transcription initiation[15,64-66]. Thus the "TATA" box alone *in vivo* may suffice to promote residual transcription levels in the absence of "CAAT"[15] or may be all that is necessary[17]. In this latter case however, removal of the first four bases of the "TATA" box produced an 80% drop in expression. Our results, suggesting that a complete or partial "TATA" box alone supports a residual level of *nos* expression, are therefore not without precedent.

The results presented in this paper represent the first functional analysis of a promoter expressed from the plant chromosome. Although there is considerable controversy over the evolutionary origins of the T-DNA and hence the *nos* gene, the genes of the T-DNA are expressed primarily within the plant nucleus, by RNA polymerase II[34,67-69]. Thus the *nos* promoter must resemble plant promoters sufficiently well for the enzyme to bind, and transcribe. The results presented here therefore have relevance to other plant genes currently under analysis.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to Professor D. Boulter for his continued support and encouragement of this research. Research funding was provided by SERC, in the shape of a project grant to CHS, and a research studentship to GHC. The longhand manuscript of this paper was decoded into typescript by Ethne Ellis. This project was performed under MAFF licences PHF 346/37 and 346/55, issued under the Plant Pests (Great Britain) Order 1980.

*To whom all correspondence should be addressed

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