Volume 253, number 1,2 167-172 FEB 07504 August 1989

# Gene transfer in plant protoplasts

## Inhibition of gene activity by cytosine methylation and expression of single-stranded DNA constructs

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Received 20 June 1989

Transfection of petunia protoplasts with the plasmid pUC118CaMVCAT, bearing the bacterial chloramphenicol acetyltransferase (CAT) gene, resulted in the appearance of CAT activity. No CAT activity was detected in protoplasts transfected with the hemimethylated form of pUC118CaMVCAT. Transfection of petunia protoplasts with ss DNA constructs bearing the coding and noncoding sequences of the CAT gene also resulted in the appearance of CAT activity, indicating expression of ss DNA constructs in transfected protoplasts. The relevance of our observations to control of gene expression in plant cells is discussed.

Gene regulation; Plant protoplast; DNA methylation

## 1. INTRODUCTION

DNA methylation has been suggested to play a central role in control of gene expression in mammalian cells [1]. Several lines of evidence support this view. A correlation between gene activity and non-methylated (hypomethylated) DNA sequences, especially in the promoter region, has been reported for various mammalian genes [2]. The inactivity of one of the two X chromosomes in cells was suggested to be partially due to DNA methylation [3] and inactivated genes can be activated by demethylation while they are still in the nucleus [4].

These studies, although establishing a correlation between hypomethylation and gene expression, do not show whether demethylation is a cause or a consequence of transcriptional activity. One way to address this question is by direct gene transfer experiments in which DNA is first methylated in vitro and then its ability to direct transcription in vivo is studied. Indeed, expression of transfected genes in animal cells was shown to be altered by introducing or removing methyl groups from their cytosine residues [5,6].

While in animal cells methylation appears to be confined to cytosine residues of the CpG sequence, in plant cells methyl groups are found on cytosine of both CpG and CpNpG palindromic sequences [7]. Also, the methylation level is higher in plant DNA than that observed in animal DNA and can be accounted for by up to 30% of the total cytosine residues in the genome [7]. Indeed, recently [8] a correlation between DNA methylation and inhibition of transcription activity of the maize transposable element Ac has been observed. This certainly may indicate that methylation and demethylation processes play an important role in the control of gene expression in plant cells. However, no direct gene transfer experiments to elucidate this question in plants have been reported so far.

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*Published by Elsevier Science Publishers B.V. (Biomedical Division)*

Here, it is shown for the first time that hemimethylated DNA constructs are not expressed in transfected petunia protoplasts. Chloramphenicol acetyltransferase (CAT) was detected in petunia protoplasts transfected with unmethylated plasmids containing the bacterial CAT gene but not in protoplasts transfected with the hemimethylated form of the same plasmids.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

XL1-Blue *E. coli* strain and VCS-M13 helper phage were obtained from Stratagene (USA); [$^{14}$C]chloramphenicol (53 mCi/mmol) from Amersham (England); M13 universal primer (17-mer) from Pharmacia (Sweden); 5-methyl-2′-deoxycytidine 5′-triphosphate (5$^{m}$dCTP) and all enzymes from Boehringer-Mannheim (FRG); and all other biochemicals were of molecular biology grade from Sigma (USA). pUC8CaMVCAT was a generous gift from Dr V. Walbot (Department of Biological Sciences, Stanford University).

### 2.2. *Preparation of petunia protoplasts*

*Petunia hybrida* protoplasts (line 3704) were obtained from exponentially growing cells by incubating cells (25%, w/v) in cpw solution [9] (10 mM $CaCl_2$, 0.2 mM $KH_2PO_4$, 2 mM $MgSO_4$, 1 mM $KNO_3$ supplemented with 10% mannitol) containing 2% cellulase R-10, 0.3% macerozyme R-10 (Yakult Pharmaceutical Industry, Nishinomiya, Japan) and 0.01% pectolyase Y-23 (Seishin Pharmaceutical, Chiba, Japan), for 15 h at 26°C in the dark.

### 2.3. *Transfection of protoplasts with plasmid DNA molecules*

Transfection of protoplasts with DNA molecules was performed essentially as described by Krens et al. [10] and Vasil et al. [11] and in our previous works [12,13]. Briefly, protoplasts were washed twice by centrifugation (300 × *g*, 5 min) in MS '150' medium [14] and resuspended in solution T (30 mM $CaCl_2$, 13% mannitol) to give a concentration of $4 \times 10^6$ protoplasts/ml. Unless otherwise specified, 30 μg double-strand plasmid DNA or 15 μg single-strand constructs, 80 μg calf thymus DNA and 0.4 ml polyethylene glycol (PEG) 1500 (40% in solution T) were added sequentially, followed by mixing and a 30 min incubation at 26°C with gentle shaking. All subsequent steps were as described in [12,13].

### 2.4. *Determination of CAT activity*

CAT activity in transfected protoplasts was determined as in [12,13]. Briefly, cells were pelleted and resuspended to give a concentration of 50% (v/v) in solution containing 0.25 M Tris-HCl (pH 7.8), 1 mM EDTA and 0.5 mM PMSF. The suspension obtained was frozen in liquid nitrogen for 5 min and thawed at 37°C for 5 min. After repeating the freeze-thawing three times, the suspension was centrifuged (10000 × *g*, 5 min), the supernatant was collected, heated for 10 min at 60°C, and after centrifugation, the supernatant was collected again. For CAT activity determination 0.6 μCi [$^{14}$C]chloramphenicol (CM) (53 mCi/mmol), 0.5 mM acetyl-CoA and 0.25 M Tris-Cl (pH 7.8) were added to the previously heated supernatant to give a final volume of 250 μl. All subsequent steps were as described before [12,13]. CAT activity is expressed as counts present in spots of the acetylated forms of CM as a percentage of the counts in spots of non-acetylated plus acetylated forms of CM.

### 2.5. *Construction and synthesis of pUC118CaMVCAT (a and b)*

pUC118CaMVCAT was constructed by subcloning the 2330 bp *Hin*dIII fragment ('expression unit') from pUC8CaMVCAT [12,13] into the polylinker of pUC118 vector [15] (fig.1). The expression unit includes the cauliflower mosaic virus (CaMV) 35 S promoter, the chloramphenicol acetyltransferase (CAT) gene and the nopaline synthase (NOS) polyadenylation sequence (fig.1). Two plasmids, pUC118CaMV CATa and pUC118CaMVCATb, were isolated bearing the expression unit in opposite orientations, so that when single-stranded molecules are produced, the expression unit is either in the coding (ss-pUC118CaMVCATb) or noncoding orientation (ss-pUC118CaMVCATa). The plasmids were propagated in XL1-Blue *E. coli* and isolated as in [16].

### 2.6. *Preparation of single-stranded (ss) DNA and in vitro synthesis of hemimethylated double-stranded (ds) DNA*

ss DNA molecules were obtained essentially as described [17]. Exponentially growing XL1-Blue *E. coli* cells (previously transformed with pUC118CaMVCATa or b) were infected with VCS-M13 helper phage ($1 \times 10^{11}$ PFU/ml) and the recombinant phages which were released into the medium were precipitated by polyethylene glycol (PEG) 8000 (20%, w/v) in 2.5 M NaCl [17]. Second-strand synthesis of the single-stranded pUC118CaMVCAT (a or b) was performed in a final volume of 30 μl of 44 mM Tris-HCl (pH 7.9), 20 mM $MgCl_2$, 13 mM DTT, 10 mM ATP, 300 μM deoxynucleotides in the presence of 5 μg ss pUC118CaMVCAT (a or b) and 17-mer M13 universal primer to give 1:100 molar ratio, respectively. To promote annealing between the universal primer and ss DNA, the mixture was allowed to incubate for 1 h at 55°C and then for 45 min at 37°C. At the end of the incubation period DNA polymerase I Klenow fragment (10 U) and $T_4$ DNA ligase (2 U) were added and the mixture was incubated at 28°C for 16 h to allow synthesis of the complementary strand. Formation of hemimethylated construct was obtained by substituting the dCTP in the reaction mixture with 5$^{m}$dCTP.

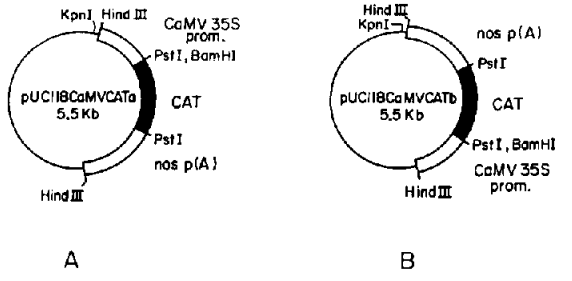


Fig.1. Schematic representation of pUC118CaMVCATa and pUC118CaMVCATb.

## 3. RESULTS

### 3.1. *The CAT gene of hemimethylated constructs is not expressed in transfected protoplasts*

The results in fig.2A show that transfection of petunia protoplasts with pUC118CaMVCAT resulted in the appearance of the CAT enzyme. It is also evident from our results that the relative orientation of the CAT expression unit (containing the CaMV 35 S promoter, the bacterial CAT gene and the NOS polyadenylation site; see fig.1) did not affect its expression efficiency. Practically the same levels of CAT activity were obtained following transfection of petunia protoplasts with plasmids containing the CAT expression unit in the two opposite orientations (fig.2A, lanes I,II).

In order to study the effect of cytosine methylation on CAT gene expression, the plasmids pUC118CaMVCATa and b (see fig.1) were hemimethylated via the procedure described in section 2. This method implies the use of ss DNA as a template for a methylated and unmethylated complementary strand [18].

Agarose gel electrophoresis clearly showed that the in vitro synthesized ds constructs were indeed hemimethylated as can be inferred from their resistance to restriction by *Pst*I (fig.2B, lanes V,VII). Also, the hemimethylated plasmids were found to be resistant to another series of restriction enzymes such as *Msp*I, *Hpa*II and *Pvu*II but were cleaved with restriction enzymes that are not sensitive to cytosine methylation such as *Taq*I and *Kpn*I (not shown) [19].

The view that the inability of *Pst*I to digest the in vitro hemimethylated ds DNA is indeed due to the presence of methyl groups and not to the experimental conditions used can be inferred from the results showing that the unmethylated in vitro synthesized ds plasmids were cleaved by *Pst*I as the original plasmid (fig.2B, lanes IV,VI).

As is evident from our results, the in vitro synthesized unmethylated ds constructs were able to induce the appearance of the CAT enzyme in transfected protoplasts (fig.2A, lanes III,IV). However, CAT activity in protoplasts transfected with the in vitro synthesized ds DNA plasmids (fig.2A) was significantly lower than that obtained following the use of the original plasmids (fig.2A, lanes I,II). Transfection of petunia protoplasts with the hemimethylated ds DNA constructs, on the other hand, did not result in any CAT activity (fig.2A, lanes V,VI; and fig.3). Previously [13], we have shown that the activity of the CAT expression unit in transfected protoplasts is highly dependent on the

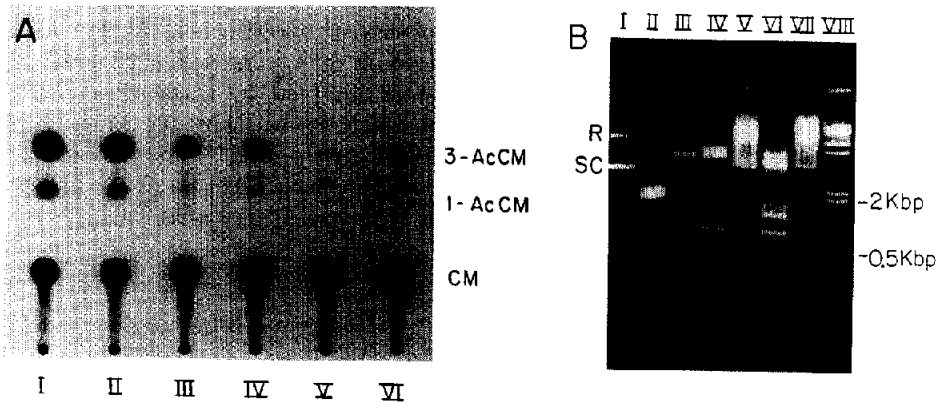


Fig.2. Characterization of the in vitro synthesized ds pUC118CaMVCAT. (A) CAT expression in transfected protoplasts. (B) Gel electrophoresis analysis. (A) Petunia protoplasts were transfected with the following DNA constructs: (I) supercoiled pUC118CaMVCATa, (II) supercoiled pUC118CaMVCATb, (III,IV) in vitro synthesized ds pUC118CaMVCATa and b, respectively; (V,VI) same as (III,IV) but of the hemimethylated structures. All other experimental conditions and CAT activity determination were as described in section 2. (B) (I) ds pUC118CaMVCATa (original plasmid, see fig.1A). The following DNA constructs were incubated with *Pst*I: (II) ss pUC118CaMVCATa, (III) ds pUC118CaMVCATa, (IV,V) non-methylated and hemimethylated constructs respectively, of the in vitro synthesized pUC118CaMVCATa, (VI,VII) same as (IV,V) but of pUC118CaMVCATb (see map in fig.1A,B); (VIII) λ DNA digested with *Hin*dIII – size markers. R, relaxed form of plasmid; SC, supercoiled form of plasmid. In all cases gel was loaded with 0.7 μg DNA. Please note that as expected, only the non-methylated ds structures, and not the ss constructs or hemimethylated ds DNA, were cleaved by *Pst*I.

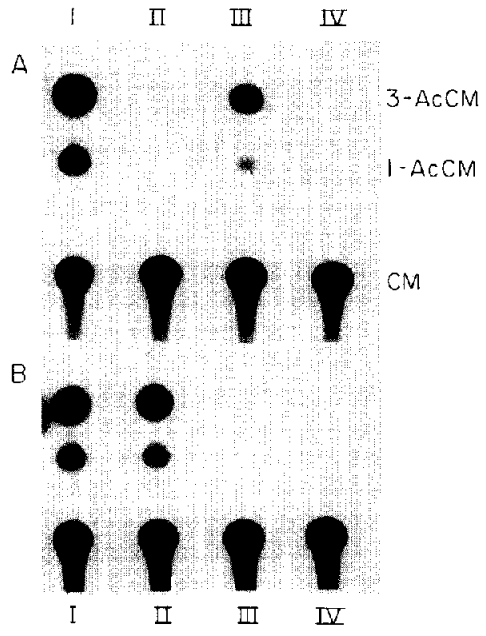


Fig.3. Transfection of petunia protoplasts with hemimethylated linearized DNA constructs: inhibition of CAT gene expression. Petunia protoplasts were transfected with: (A) (I) in vitro synthesized linear ds pUC118CaMVCATa; (II) as (I) but plasmid was cleaved with *Bam*HI; (III) ss pUC118CaMVCATa treated with *Bam*HI; (IV) control calf thymus DNA. (B) (I,II) In vitro synthesized linear pUC118CaMVCAT (a,b, respectively); (III,IV) same as (I,II) but for the hemimethylated structures. All experimental conditions including in vitro (second strand) synthesis of ds DNA structures, hemimethylation, transfection of protoplasts and CAT activity determination were as described in section 2. The non-methylated and hemimethylated plasmids were linearized by restriction with *Kpn*I (see fig.1).

topology of the DNA constructs used, the linear form being much more active than the supercoiled form. Furthermore, transfection with the relaxed forms led to very low levels of CAT activity [13]. Gel electrophoresis analysis showed (fig.2B), as expected, that the in vitro synthesized constructs are in a relaxed form. Therefore, it is not surprising that their introduction into petunia protoplasts resulted in only a low level of CAT activity. A way to increase their level of expression was to convert them into their linear forms. The results in fig.3A show that transfection of petunia protoplasts with the unmethylated linearized structures of the in vitro synthesized plasmids resulted in a high level of CAT expression. Linearization did not alter the properties of the pUC118CaMVCATa plasmid and restriction of the linearized form with *Bam*HI resulted in inactivation of the CAT gene (fig.3A, lane II, and see also map for *Bam*HI restriction site in fig.1). The same results were obtained following the use of pUC118CaMVCATb. The results in fig.3B show that high CAT activity was observed following transfection of protoplasts with the in vitro synthesized linear plasmids containing the CAT expression unit in the two opposite orientations (fig.3B, lanes I,II). No CAT activity, however, could be detected following transfection of petunia protoplasts with the two hemimethylated linearized structures (fig.3B, lanes III,IV).

### 3.2. *ss DNA constructs are active and can support gene expression in plant protoplasts*

The use of in vitro synthesized ds DNA plasmids forced us to employ as a control in transfection experiments ss DNA constructs which served as a template for the complementary strand synthesis. Surprisingly, the introduction of ss DNA (pUC118CaMVCATa or b) constructs into petunia protoplasts also resulted in the expression of the CAT gene. This is evident from the results in fig.3A (lane III) and table 1 which show that transfection of petunia protoplasts with the ss DNA constructs resulted in the appearance of CAT activity. Interestingly, transfection with a single-DNA strand containing the noncoding sequence resulted in a somewhat higher activity than that observed following transfection with a strand containing the coding sequence (table 1). The expression observed by transfection with the ss DNA preparation was not due to contamination by ds DNA as inferred from the results showing that restriction with *Bam*HI of the ss DNA preparation did not practically affect its activity (table 1). On

Table 1

Expression of ss DNA constructs in transfected petunia protoplasts

| DNA construct | Acetylated CM (%) |
|---|---|
| ds-pUC118CaMVCATa | 11.2 |
| ds-pUC118CaMVCATa-*Bam*HI | <0.3 |
| ss-pUC118CaMVCATa | 8.5 |
| ss-pUC118CaMVCATa-*Bam*HI | 7.3 |
| ss-pUC118CaMVCATb | 7.1 |
| ss-pUC118CaMVCATb-*Bam*HI | 5.4 |

All experimental conditions were as described in section 2 and the legend to fig.3. Percentage of acetylated CM was measured as described in the text

the other hand, as expected, restriction with *Bam*HI abolished completely the expression of the ds DNA (fig.3A and table 1).

## 4. DISCUSSION

The present experiments clearly show that hemimethylated DNA constructs are not expressed in transfected plant protoplasts. This is evident from our experiments showing that transfection of petunia protoplasts with hemimethylated plasmids did not lead to the appearance of CAT activity as opposed to transfection with unmethylated constructs of the same plasmids. The inhibition reported is characteristic of the behavior of methylated expression unit of animal genes and was observed following transfection of animal cells with plasmids containing genes such as the human $\beta$-globin or Herpes Simplex thymidine kinase (TK) genes [20,21].

The use of hemimethylated DNA constructs does not allow studies on the question of whether the inactivation of the CAT gene is due to methylation of specific sites. However, indirect evidence supports the view that inhibition of gene expression observed in such systems is not due to unspecific alteration of the DNA structure. This is inferred from previous experiments showing that transfection of animal cells with similar hemimethylated DNA constructs resulted in integration of the transfected DNA into the chromosomal DNA [20]. Furthermore, in those experiments the integrated hemimethylated DNA was subjected to the process of DNA replication as any other chromosomal DNA sequences [20]. Our present results also show that both the relaxed and the linear forms of the hemimethylated plasmids were not expressed. This certainly shows that inhibition of expression was independent of the DNA form. The CAT gene was shown to be inactive in protoplasts transfected with plasmids containing methylcytosine residues in their coding or noncoding strands showing that inhibition via methylation is not strand specific.

In order to characterize further the behavior of the hemimethylated DNA structure in plant cells, we are currently attempting to study stable transformation with hemimethylated DNA constructs. The function and fate of such hemimethylated sequences will be analyzed.

Results of the present work also show that transfection of plant protoplasts with ss DNA constructs containing the bacterial CAT gene resulted in the appearance of CAT activity. The ability of externally added ss DNA constructs to support synthesis of specific products in microinjected oocytes and transfected animal cell cultures has been reported before [22,23]. To the best of our knowledge, the present work is the first to demonstrate the activity of ss DNA in plant protoplasts. It has been previously observed in microinjected oocytes [22] that ss DNA serves as a template for the synthesis of a complementary strand resulting in the appearance of ds DNA. This newly synthesized ds DNA serves as a substrate for the RNA polymerase. The ability of the noncoding ss DNA constructs to induce CAT activity in transfected protoplasts indicates that a complementary strand is also synthesized in the present system. If true, the present system may serve as an experimental tool to study various aspects of DNA synthesis and its replication in plant cells.

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