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Biological activity of hemimethylated and single-stranded DNA after direct gene transfer into tobacco protoplasts

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In this investigation we analyzed the effect of DNA methylation on gene expression in tobacco protoplasts. Methylation of the chimeric GUS gene (CaMV 35s/GUS/NOS) with the *Hpa*II methyltransferase had no effect on transient and on stable GUS gene expression. The chimeric gene was then cloned as single-stranded DNA. In vitro second-strand DNA synthesis was performed with 5-methylcytosine, leading to methylation of every cytosine in one DNA strand. After transfection this DNA was totally inactive. Control experiments revealed that the single-stranded DNA was as active as the double-stranded form.

Hemimethylation; Plant protoplast; Gene expression; DNA, single-stranded; DNA replication

1. INTRODUCTION

Changing of the DNA methylation pattern is considered as an important step in the regulation of gene expression by mammalian cells. The current concept proposes that undermethylation of DNA is required for gene activation. Direct evidence that DNA methylation can block gene expression was obtained by transfection of in vitro methylated DNA into tissue culture cells (review [1]). In mammalian cells, cytosine within the CpG dinucleotide sequence is the only target for the cellular methyltransferase and the extent of methylation is in the range of 3-5%. In the nuclei of plant cells, the extent of DNA methylation is significantly higher. Up to 30% of all cytosines can be methylated. The methylation pattern is distributed between the sequences CpG and CpXpG [2]. There is some evidence that in plants, DNA methylation correlates with gene inactivation. It was demonstrated that Ti-DNA from *Agrobacterium* in transformed plant tissues can become methylated [3]. Methylation correlated with a low expression rate of the nopaline synthase gene (NOS). Treatment of the cells with 5-azacytidine caused demethylation and an increased transcription rate of the Ti-DNA, indicating that DNA methylation can inactivate plant genes [4]. An inverse correlation between gene activity and DNA methylation was also described for the maize transposable element Ac [5].

Here, we investigated whether in vitro DNA methylation can block gene expression in tobacco protoplasts. Therefore, we methylated the chimeric GUS gene (CaMV 35s/GUS/NOS) with the bacterial *Hpa*II methyltransferase. After transfection of the methylated DNA into tobacco protoplasts we found that *Hpa*II methylation was not sufficient to cause GUS inactivation. However, inactivation of the GUS gene was observed after transfection of hemimethylated DNA where all cytosine residues of one DNA strand were replaced by 5-methylcytosine. The hemimethylated DNA was obtained by second-strand synthesis of the M13 cloned CaMV 35s/GUS DNA. Control transfection experiments with the single-stranded (ss) DNA revealed a very unexpected result, namely that both the coding and non-coding ss DNA of the

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CaMV 35s/GUS DNA were highly active after transfection into plant protoplasts.

2. MATERIALS AND METHODS

2.1. *Plant material*

Nicotiana tabacum cultivar SRI was grown as sterile shoot culture at 26°C in a cycle of 16 h light and 8 h dark. Protoplasts were isolated from 6-week-old leaves according to [6]. PEG-mediated transfection was performed as in [7].

2.2. Hpa*II methylation*

The pBI221.2 DNA was methylated in vitro with *Hpa*II methyltransferase as described [8]. Completeness of methylation was assessed by treatment with *Hpa*II and *Msp*I restriction endonucleases and agarose gel electrophoresis. Before transfection pBI221.2 was linearized by *Nde*I which cuts outside the CaMV 35s/GUS/NOS gene.

2.3. *Hemimethylation*

The 3 kbp *Bam*HI/*Hin*dIII fragment (fig.1), containing the CaMV 35s promoter, the GUS gene and the NOS polyadenylation signal, was isolated from pBI221.2 and ligated into the corresponding polylinker sites of the RF-M13 DNA. Preparation of ss M13 DNA (pM13-GUS I) and second-strand synthesis (M13-GUS II) were performed as in [9]. For transfection the double-stranded M13 vector was cut with *Dra*I.

2.4. *Expression assay*

At different times after transfection, regenerating protoplasts were collected by centrifugation. After sonication, the cell lysates were assayed for GUS activity according to [8]. GUS activity was determined as relative fluorescence per protein content. Protein concentration was determined using the Bio Rad assay.

3. RESULTS AND DISCUSSION

3.1. *Biological activity of* Hpa*II methylated pBI221.2 DNA*

The pBI221.2 DNA, containing the chimeric GUS gene, was methylated in vitro by the M · *Hpa*II methyltransferase as described in section 2. The biological activity of the methylated and mock-methylated DNA was tested following transfection into tobacco protoplasts. At various times after DNA transfer, regenerating protoplasts were

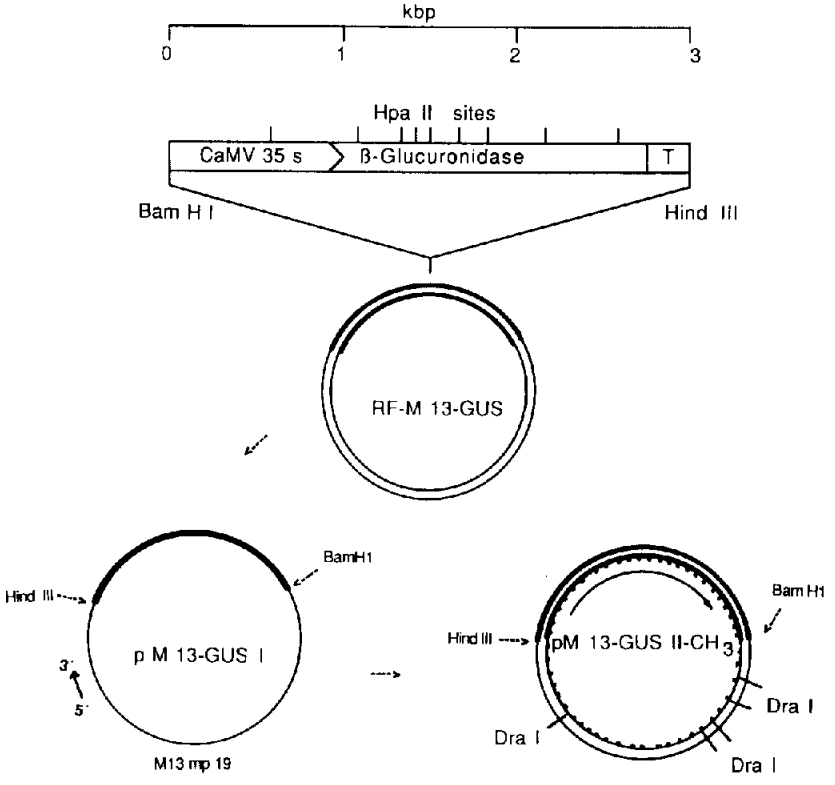

Fig.1. The upper part shows the RF-M13 mp19 DNA with the chimeric gene (RF-M13 GUS). The single-stranded pM13-GUS I DNA, isolated from the phages was used to synthesize the hemimethylated DNA (pM13-GUS II-CH_3).

lysed and the GUS enzyme activity was determined. We observed no inhibition of GUS gene expression by M·*Hpa*II methylation. As summarized in fig.2, the biological activity of the methylated DNA was always similar to the activity of mock-methylated DNA. To determine whether M·*Hpa*II methylation blocks GUS gene expression in calli, we cotransfected methylated and unmethylated pBI221.2 DNA, respectively, together with the pHP23 DNA (mediates kanamycin resistance) [10] into tobacco protoplasts. About 2 months after transfection, kanamycin-resistant calli were harvested and GUS enzyme activity was analyzed. Again there was no indication that *Hpa*II methylation caused inactivation of the GUS gene. Since the CaMV 35s promoter cannot be methylated by the M·*Hpa*II enzyme within essential sequences [11] and the structural part of the GUS gene contains only 8 *Hpa*II sites, undermethylation may be the reason why inhibition of GUS gene expression did not occur. Therefore, we decided to replace every cytosine of one DNA strand by 5-methylcytosine to ascertain whether this heavily methylated DNA was still biologically active in tobacco protoplasts. That hemimethylation can block gene expression in mammalian cells was shown recently [9].

3.2. *Biological activity of hemimethylated DNA*

In order to obtain the chimeric GUS gene in a hemimethylated form, the *Bam*HI/*Hin*dIII fragment was isolated from the pBI221.2 DNA and inserted into the corresponding sites of the ds RF-M13 DNA. After propagation in *E. coli* the ss DNA (pM13-GUS I) was isolated from the phages and used as a DNA template for in vitro second-strand DNA synthesis. In this way the coding DNA strand was either synthesized in the methylated (pM13-GUS II-CH_3) or non-methylated form (pM13-GUS II). The double-stranded DNA was transfected into tobacco protoplasts and GUS activity was assayed. As summarized in fig.3, the hemimethylated DNA was biologically inactive at each test point. The non-methylated pM13-GUS II DNA exhibited activity similar to that of pBI221.2 DNA (fig.2). Interestingly, a strong reduction in GUS activity was also observed by methylation of the non-coding DNA strand (not shown). These results are in accordance with our earlier observations, namely that hemimethylation causes inactivation of the HSV-TK chromatin after microinjection into rat2 cells [9]. Further experiments are required in order to establish whether methylation of the promoter sequence or of the structural gene part alone is sufficient to cause gene inactivation.

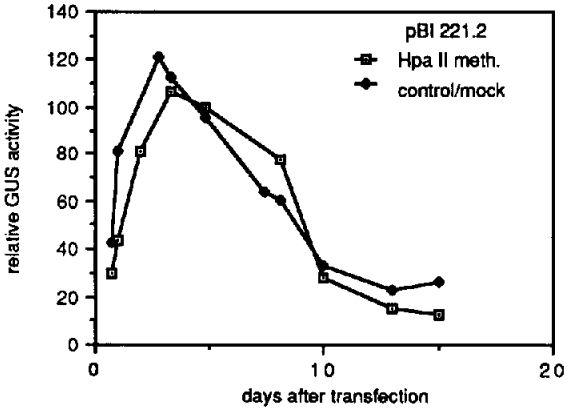

Fig.2. Relative GUS activity after transfer of M·*Hpa*II methylated and non-methylated pBI221.2 DNA. For each experiment 0.5×10^6 protoplasts were transfected with 1 μg *Nde*I-linearized pBI221.2 DNA.

3.3. *Biological activity of single-stranded DNA in tobacco protoplasts*

Our experiments also included the transfection

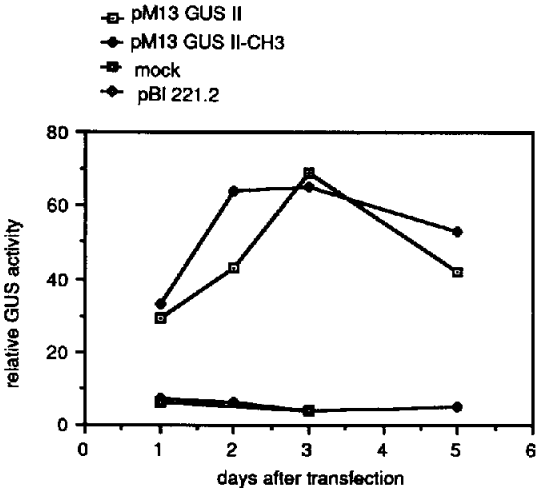

Fig.3. Relative GUS activity after transfer of the hemimethylated pM13-GUS II-CH_3, the non-methylated pM13-GUS II and pBI221.2 DNA. For each experiment 0.5×10^6 protoplasts were transfected with 1 μg linearized DNA. The 'mock' values represent GUS activity of protoplasts transfected with calf thymus DNA only.

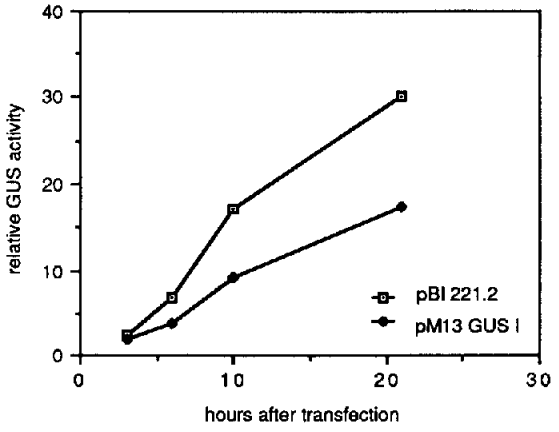

Fig.4. Relative GUS activity after transfer of single-stranded pM13-GUS I DNA (non-coding strand) and pBI221.2 DNA. For each experiment 0.5×10^6 protoplasts were transfected with 1 μg DNA.

of ss pM13-Gus I DNA into tobacco protoplasts. From these experiments we obtained an unexpected result, namely that ss DNA was highly active in the recipient cells. Irrespective of whether the coding or non-coding DNA strand was transfected, GUS gene expression occurred with nearly the same efficiency as after transfection of the double-stranded pBI221.2 DNA. From these observations we conclude that the single-stranded DNA is converted into double-stranded DNA before transcription. Since GUS activity was observed as early as 6 h after DNA transfer, synthesis of the second DNA strand must have occurred before the plant cells entered the S-phase (our observation). Additional experiments must be performed to prove that the single-stranded DNA is indeed converted into double-stranded molecules at such an early time.

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