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Increasing loop domain size does not diminish effects of matrix attachment regions on transgene expression in tobacco cells in culture

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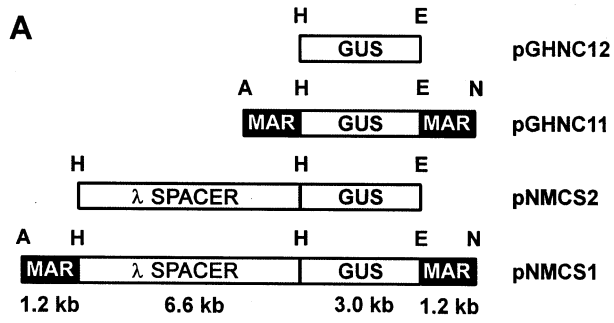
It is now widely held that the chromatin of eukaryotic organisms is organized into loop domains in which chromatin fibers are attached to the nuclear matrix by specific interactions of nuclear matrix proteins with DNA sequences called matrix attachment regions (MARs) [1,2]. Several MARs have been isolated and incorporated into constructs used for transformation. Work with animal cell culture systems has consistently shown much higher average levels of reporter gene expression in cell lines in which reporter genes are flanked with cloned MARs than in control lines without flanking MARs [1]. MARs have similar effects in plant cell systems. For example, in a tobacco cell culture system, we have shown that a heterologous MAR (a yeast MAR that binds weakly to the tobacco nuclear matrix) increases reporter gene expression by 12-fold, and a strong matrix-binding tobacco MAR increases reporter gene expression by 60-fold [3].

The mechanisms by which MARs increase reporter gene expression are unknown. MARs do not appear to act as typical enhancers, as they have little effect in transient expression assays [1,2]. Most proposed mechanisms involve chromatin structure [1,2]. According to one model, MARs affect transgene expression by creating independent, topologically isolated domains. The transgenes in these independent domains are insulated from chromatin structure of the native domains (either transcriptionally active or repressed) into which they become incorporated. If transgenic MARs do act by creating independent domains containing the transgene, the question remains of why the independent domains have a transcriptionally active chromatin structure. We have previously speculated that independent domains created by cloned MARs may have transcriptionally active chromatin structures because they are too small to form stable, transcriptionally repressed, condensed chromatin fibers in vivo [2,3]. In our previous experiments, the putative domain formed by cloned MARs contains 3 kb of DNA, enough to form 16 plant nucleosomes. Even fewer nucleosomes may form on the transgenes in vivo, as close proximity to the nuclear matrix may sterically inhibit nucleosome formation. As the structure of the 30-nm chromatin fiber is not well understood, the number of nucleosomes necessary to form a stable, transcriptionally repressed structure is unknown. Formation of folded chromatin fibers presumably is dependent upon nucleosome–nucleosome interactions. If we consider the solenoid model of the 30-nm fiber (six nucleosomes per turn of the solenoid), it is logical to assume that a minimum of 12 nucleosomes would be required to form a stable, folded structure. This would allow each nucleosome to interact with at least one nucleosome other than its linear neighbors. Further turns of the solenoid would be expected to further stabilize the structure. For example, in a structure containing 18 nucleosomes (three turns of the solenoid), the nucleosomes of the middle turn could interact with nucleosomes of the outside two turns of the solenoid. Carruthers et al. [4] have provided evidence that a reconstituted linear DNA fragment containing 12 nucleosomes can form a stable, folded structure, but the relationship of this structure to the 30-nm fiber is unclear. The work of Butler and Thomas [5] indicates that more nucleosomes may be required. These workers observed a change in the hydrodynamic properties of native, rat liver nucleosome oligomers above the size of 50 nucleosomes. They attributed the change to higher-order folding.

The considerations mentioned above suggest that in our previous work [2,3] the 16 nucleosomes that would be expected to form on 3 kb of DNA bounded by MARs may be below or near the minimum required to form stable, folded chromatin fibers in vivo. Based on these ideas concerning the stability of higher-order structure in chromatin, we have asked if the enhancement of transgene expression by MARs can be counteracted by increasing the amount of DNA in the putative loop domain to a point at which a stable condensed chromatin fiber could be formed.

In order to answer this question we have transformed plant cells in culture by microprojectile bombardment as we have previously described [3]. A co-transformation procedure is used in which a selectable marker (NPTII conferring kanamycin resistance) is carried on a separate plasmid from the reporter gene. As in our previous experiments we have used the reporter plasmids, pGHNC11 and pGHNC12 (Fig. 1A). These plasmids contain the GUS reporter gene cassette flanked by the RB7-6 tobacco MAR and a control of the GUS cassette without flanking MARs. We also used a plasmid (pNMCS1 in Fig. 1A) in which λ DNA has been inserted between the GUS reporter cassette and the MAR. This 'spacer' DNA would increase the size of the putative MAR-bounded loop domain to 52 nucleosomes. The λ DNA (a 6.6-kb *Hin*dIII fragment, nucleotides 37586–44141) has an AT content of 51% and does not bind to the tobacco nuclear matrix (data not shown). A control plasmid (pNMCS2) with the λ DNA and the GUS reporter cassette but no MARs was also used.

Kanamycin-resistant transformed cells were grown in liquid culture for 2 months with transfers every 7 days. At this time, cells were harvested and protein extracts were made in order to measure GUS specific activity [3]. Fig. 1B shows the results of the GUS specific activity measurements. Increasing the amount of DNA in the putative independent domain to a size that would support 8.6 turns of a nucleosome solenoid does not diminish the effect of MARs on enhancing reporter gene expression. The specific activity of the MAR-SPACER-GUS-MAR transformed cell lines is slightly higher than that in the MAR-GUS-MAR lines, but the difference is not statistically significant. In the lines transformed with constructs lacking MARs, the GUS activity was slightly lower in the



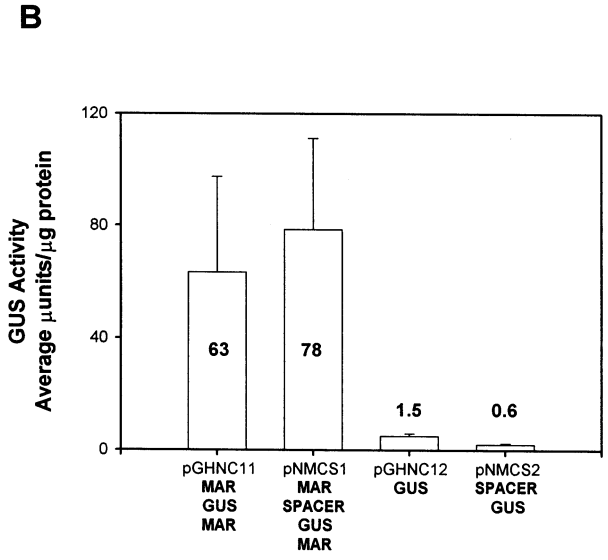


Fig. 1. A: Inserts in plasmids used in the transformation of tobacco NT-1 cells by microprojectile bombardment. All plasmids contain the reporter gene *GUS* (β-glucuronidase) cassette. The top plasmid has the *GUS* cassette alone. In the next plasmid, the *GUS* cassette is flanked by the 1.2-kb tobacco RB7-6 MAR oriented as direct repeats. In the third plasmid a 6.6-kb λ DNA fragment (spacer) is located 5′ to the *GUS* cassette. In the last plasmid the spacer fragment is placed between the 5′ MAR and the *GUS* cassette. All inserts are cloned into pBluescript II SK+ at the indicated sites. A, *Apa*I; E, *Eco*RI; H, *Hin*dIII; N, *Not*I. B: Effect of increasing putative domain size on MAR enhancement of GUS transgene activity in tobacco cells in culture. The average GUS specific activity is plotted for tobacco cell lines transformed by plasmids with the inserts shown in A. The specific activities are higher in both lines in which the *GUS* gene is flanked by MARs than in controls in which the *GUS* gene is not flanked by MARs (95% confidence). Inclusion of 6.6 kb of λ DNA as a 'spacer' between the 5′ MAR and the *GUS* gene or between the plasmid backbone and the *GUS* gene had no effect on GUS specific activities. The differences between specific activities in the MAR-GUS-MAR and MAR-SPACER-GUS-MAR and between specific activities in the GUS and SPACER-GUS cell lines are not significantly different. The numbers of cell lines in each group are as follow: MAR-GUS-MAR = 30, MAR-SPACER-GUS-MAR = 28, GUS = 27, SPACER-GUS = 21.

SPACER-GUS lines than in the GUS-only lines, but again, this difference was not statistically significant.

Because most DNA constructs used to test the effects of MARs on transgene expression contain amounts of DNA between the MARs that would be sufficient to form only a few nucleosomes [1–3], we hypothesized that the mechanism of MAR activity is the formation of loop domains that are too small to form a stable, transcriptionally repressed chromatin fiber. Increasing the amount of DNA in the putative MAR-bounded loop domains to a size that would accommodate 52 nucleosomes (nearly nine turns of a nucleosome solenoid) does not diminish the MAR-mediated enhancement of transgene expression in tobacco cells in culture. In vitro data indicate that 52 nucleosomes would be more than enough to support a stable, condensed chromatin fiber [5]. Thus, the explanation for MAR enhancement of transgene expression must lie elsewhere.

We also note that the 5′ MAR is able to influence transgene expression at a fairly great distance (at least 6.6 kb from the promoter). It could be argued that the MAR located 3′ to the transgene is by itself causing the enhancement of transgene expression. But we have previously shown [2,3] that both 5′ and 3′ flanking MARs are necessary to get the full MAR effect on increasing transgene expression.

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