Update on Molecular Biology

Nuclear Matrix Attachment Regions and Transgene Expression in Plants

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DNA sequences called matrix attachment regions (MARs) or scaffold attachment regions (SARs) have recently attracted much attention because of their perceived capacity to increase levels of transgene expression and reduce transformant-to-transformant variation of transgene expression in both plants and animals. Work with these sequences is in its early stages and data that seem to be contradictory have been presented. We do not intend to resolve these controversies here (this will be accomplished by further research). Rather, we will discuss the hypothesized role of MARs in chromatin structure, how MARs are isolated and characterized, what effects MARs have had on the expression of transgenes and the models that have been evoked to explain those effects.

THE ROLE OF CHROMATIN STRUCTURE IN GENE EXPRESSION

All models that attempt to explain how MARs can affect transgene expression deal with the regulation of gene expression at the chromatin structure level. We cannot review the role of chromatin structure in any detailed manner here, but many extensive reviews have been written concerning this subject (see Paranjape et al., 1994, and reviews referenced therein; Reeves, 1984). In brief, control of gene expression at the chromatin structure level involves access of RNA polymerase and transcription factors to their binding sites on DNA. These binding sites can be inaccessible because of the highly compact structure of chromatin fibers. A partial unfolding to a less compact, more open structure can render the binding sites accessible. A common way to depict this concept is to represent transcriptionally inactive chromatin as a compact, inaccessible 30-nm fiber and transcriptionally poised chromatin as an open, unfolded 11-nm or nucleosome fiber as shown in Figure 1A. Such depictions are undoubtedly oversimplified (van Holde and Zlatanova, 1995) but are nevertheless useful. One of the hallmarks of unfolded, transcriptionally poised chromatin is heightened general sensitivity to the endonuclease DNase I (Weintraub and Groudine, 1974). It has long been known that the DNase I sensitivity extends far beyond the immediate region of the transcribed gene

and that "domains" of transcriptionally poised chromatin exist (Stalder et al., 1980). In at least some cases the domains of transcriptionally poised chromatin (as assayed by DNase I sensitivity) correspond to structural domains in chromatin that have been described as "loop domains" (reviewed by Bonifer et al., 1991).

LOOP DOMAINS IN CHROMATIN STRUCTURE

The idea of loop domains is familiar, dating back to early observations of "lampbrush" chromosomes in amphibians. In most current models of chromatin structure, loop domains represent the third level of DNA organization. In the first level of organization, DNA is wrapped in 1¾ turns around the histone octamer to form nucleosomes and the 11-nm nucleosome fiber. The second level of organization involves the folding of nucleosome fibers into a higher order structure, the 30-nm chromatin fiber. Probably the most well-known model for this folding is the "solenoid model" (Fig. 1) that appears in many textbooks. It should be noted, however, that our knowledge of chromatin structure at this level is imprecise, and it is likely that no completely uniform structure exists for what we recognize as the 30-nm chromatin fiber (van Holde and Zlatanova, 1995). In the next higher level of organization loop domains are postulated to form by attachment of the DNA sequences (called MARs or SARs) to the proteinaceous network of filaments that runs throughout the nucleus and has been called the nuclear matrix or nuclear scaffold. MARs are approximately 1 kb, and the loop domains between MARs range from about 5 to 200 kb (Pienta et al., 1991). For example, in the two MAR-bounded domains shown in Figure 1A, the unfolded, transcriptionally active loop domain on the left contains 37 nucleosomes at approximately 200 bp of DNA per nucleosome for a 7.4-kb domain. The highly coiled, transcriptionally inactive domain on the right is depicted as a solenoid of 6 nucleosomes per turn. The approximately 17 turns of the solenoid contain 102 nucleosomes or 20.4 kb of DNA.

Although the nuclear matrix has been studied for quite some time (Berezney and Coffey, 1974), its very existence has often been questioned (reviewed by Jack and Eggert,

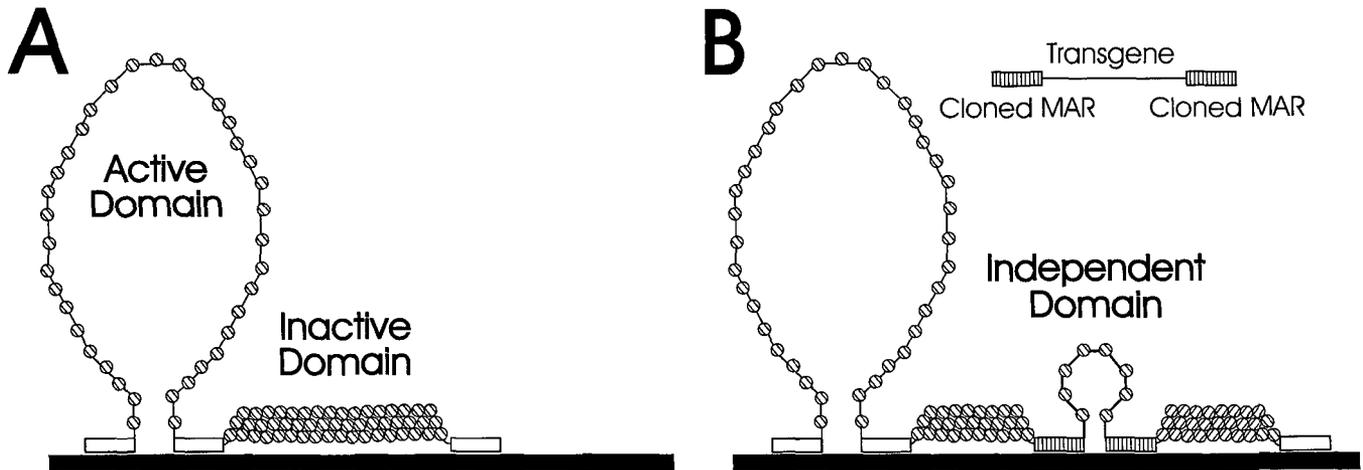* Corresponding author; e-mail steven_spiker@ncsu.edu; fax 1–919–515–3355.

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Figure 1. Models depicting the organization of chromatin into active and inactive loop domains and the formation of independent transgenic loop domains. A, MAR sequences (open boxes) interact with nuclear matrix fiber (filled bar) to form two loop domains. The active domain is depicted as an 11-nm nucleosome fiber and the inactive domain as a 30-nm fiber formed by supercoiling of the 11-nm fiber. B, An independent domain formed by the integration of MAR-flanked transgene into the inactive domain.

1992). An artist's representation of an electron micrograph of the nuclear matrix is shown in Figure 2A. The actual electron micrograph is of a nucleus from which membranes have been removed by detergent treatment and chromatin has been removed by high-salt extraction and endonuclease digestion (Capco et al., 1982). The fibers remaining after this treatment represent the nuclear matrix or scaffold. Chromatin loops are presumed to be attached to these matrix fibers by protein-DNA interactions with MARs.

To make chromosomes, loop domains presumably must be further packaged. Thus, it is not surprising that metaphase chromosomes appear to be organized on a similar fibrous network. Studies in Laemmli's laboratory have shown that when histones and most other proteins are extracted from metaphase chromosomes a "ghost" of pro-

tein remains (the chromosome scaffold), which retains the recognizable form of metaphase chromosomes. Because coiling restraints are lost when histones are removed, the DNA expands to form a cloud or "halo" around the metaphase chromosome ghost. Figure 2B is an artist's representation of a portion of such a halo from an electron micrograph by Paulson and Laemmli (1977). A portion of the chromosome scaffold can be seen with loops of DNA emanating from it. The DNA in contact with the chromosome scaffold represents MARs.

HOW MARS ARE ISOLATED AND CHARACTERIZED

MARs (SARs) are isolated and defined on an operational basis according to their affinity for the nuclear matrix
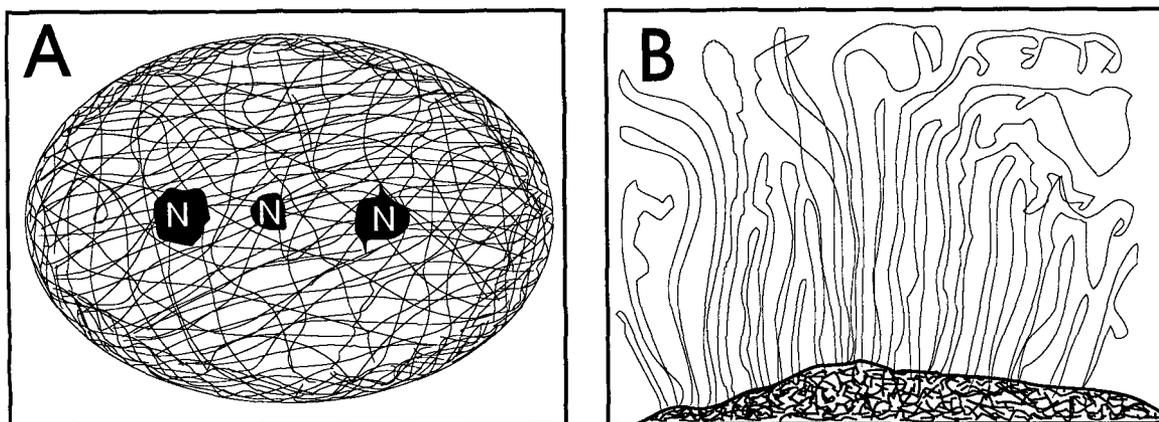

Figure 2. Nuclear matrix and halo. A, Drawing based on an electron micrograph (Capco et al., 1982) of a nucleus from which the nuclear membrane has been removed by detergents and chromatin removed by high-salt extraction, leaving the fibers of the nuclear matrix. N, Nucleoli. B, Drawing based on electron micrograph of a portion of a metaphase chromosome (Paulson and Laemmli, 1977) from which chromatin proteins have been extracted. Extraction of histones removes coiling restraints, allowing DNA to spill out and form a halo. DNA loops are presumed to be attached by MARs to the chromosome scaffold (below).

(nuclear scaffold) as shown in Figure 3. The procedure starts with isolated nuclei. In the first step the nuclear membrane and histones are removed. The distinctions between the terms nuclear matrix versus nuclear scaffold and MARs versus SARs are based on the methods of removing histones. When high salt is used, the resulting structure has been called the nuclear matrix (Berezney and Coffey, 1974). When lithium diiodosalicylate is used, nuclear scaffolds result (Mirkovitch et al., 1984). DNA sequences that bind to these structures are, of course, called MARs or SARs, depending on which procedure of isolation is used. The distinction between matrix and scaffold is gradually being lost
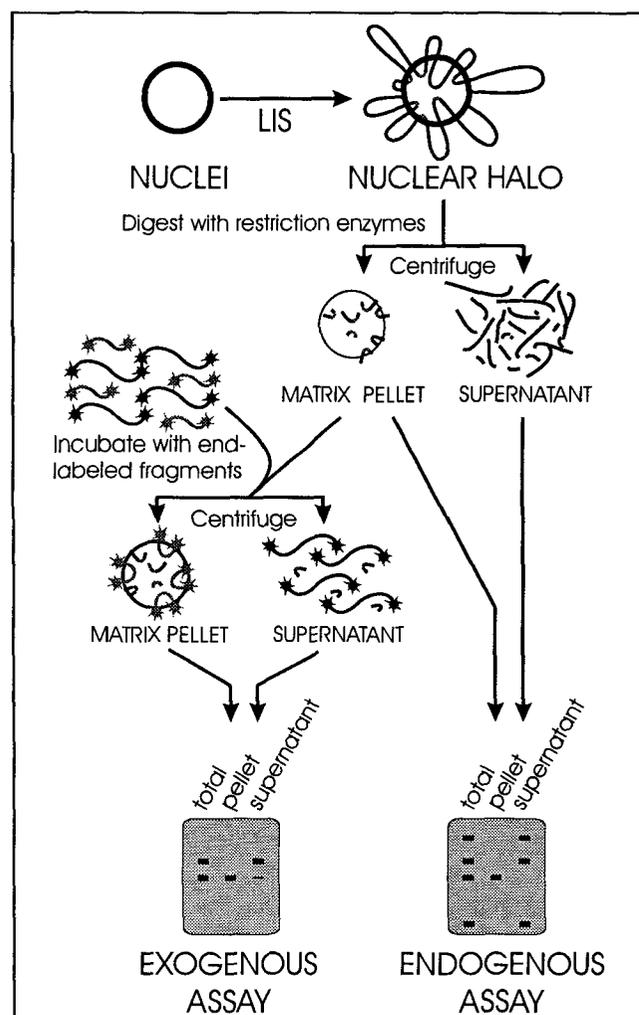
in the scientific literature. This is probably appropriate because the nuclear matrix and the nuclear scaffold are very similar by most criteria and certainly the biological entity they are intended to represent is the same. In the past we have used the terms scaffolds and SARs in work from our laboratories because we have used lithium diiodosalicylate in our procedures. We believe, however, that it is preferable to use a single term, and because the terms matrix and MARs appear to be more prevalent, we have used those terms here.

After histones have been removed, the DNA loses its coiling restraints and spills out to form a nuclear halo, as shown in Figure 2B. At this point, the nuclear halos are treated with restriction enzymes, which releases and solubilizes the DNA in the loop domains, leaving the DNA associated with the insoluble nuclear matrix (MARs by operational definition). The DNA in the loop domains and the MARs associated with the nuclear matrix can then be separated by centrifugation. Two assays are commonly used to determine whether any particular DNA sequence contains a MAR. In the endogenous assay shown in the lower right portion of Figure 3, DNA from the pellet fraction (matrix) and the supernatant fraction are purified, separated by electrophoresis, blotted, and probed with a radioactively labeled DNA fragment containing the sequences to be tested. Fragments from the pellet fraction hybridizing to the probe contain a MAR (one fragment in the figure). Fragments from the supernatant hybridizing to the probe do not contain MARs (three fragments in the figure). (Note: To get the result shown in the figure, the probe must be large enough to contain sequences that would hybridize with all four fragments shown in the "total" lane.) This assay is called the endogenous assay because it is designed to analyze DNA sequences that are present in nuclei used to make the nuclear matrices. The exogenous assay is not so limited. In this assay DNA from any source can be tested for its affinity for the nuclear matrix.

The exogenous assay is shown in the lower left portion of Figure 3. The isolated nuclear matrix is incubated with end-labeled DNA fragments (including the sequences to be assayed) along with unlabeled competitor DNA. One or more control fragments are included in this incubation. The vector used to clone the sequence in question is a convenient control. During the incubation, MAR sequences associate with the nuclear matrix either by binding to free sites or by displacing resident MARs. Sequences that bind to the insoluble nuclear matrix (operationally defined MARs) are then separated from nonbound fragments by centrifugation, and the DNA fragments from both fractions are subjected to electrophoresis and analyzed directly by autoradiography. The diagram shown in Figure 3 is typical for what we see when we use genomic DNA as competitor and run equal proportions of the pellet and supernatant fractions on the gel. This approach allows us to determine the relative binding activity of any MAR sequence for the nuclear matrix. MARs that are found almost entirely in the pellet fraction are "strong" MARs (as depicted in Fig. 3). MARs with lower proportions found in the pellet fraction are weaker. We have isolated and characterized several plant MARs using the exogenous assay and by cloning

Figure 3. Isolation of the nuclear matrix and characterization of MARs by exogenous and endogenous assays. Chromatin proteins are extracted from isolated nuclei (we use lithium diiodosalicylate, LIS), thus removing coiling restraints and allowing DNA to spill out and form a nuclear halo (compare to Fig. 2B). DNA in loop domains is solubilized by restriction enzyme digestion, resulting in formation of the nuclear matrix (compare to Fig. 2A). In the endogenous assay, DNA from the supernatant and DNA remaining with the insoluble matrices are purified, separated by electrophoresis, blotted, and probed with a sequence to be tested. In the exogenous assay, the capacity for exogenous, end-labeled fragments to bind to purified nuclear matrices is determined. See text for details.

DNA fragments co-isolating with the nuclear matrix. More detailed procedures for the isolation and assays can be found in Hall et al. (1991), Allen et al. (1993), and Hall and Spiker (1994). As a note of refinement, we emphasize that we have used the terms "endogenous" and "exogenous" here rather than "in vivo" and "in vitro." Both assays are actually in vitro assays and can indicate only affinity for the nuclear matrix, not whether any particular sequence actually is bound to the nuclear matrix and serves as an anchor for loop domains in vivo. Even in the endogenous assay, a sequence not actually bound to the nuclear matrix in vivo but with an affinity for the nuclear matrix could associate with the matrix during preparation and thus be found in the pellet fraction.

What determines whether a DNA sequence will bind specifically to the nuclear matrix? The answer to that question is presently unknown. MARs are typically very rich in AT (more than 70%) and several short "consensus sequences" have been proposed. All of the consensus sequences are essentially different arrangements of A and T and might be expected to occur frequently by chance in highly AT-rich DNA. The presence of any of the consensus sequences does not ensure that a DNA fragment will bind to the nuclear matrix, and none of the consensus sequences are indispensable (reviewed by Bode et al., 1992).

Just as we do not know precisely what determines whether a DNA sequence is a MAR, we also have little knowledge of what proteins in the nuclear matrix are responsible for binding MARs. If there is one protein that is universally responsible, that protein has not been identified. Several proteins have, however, been shown to interact specifically with MARs in vitro (von Kries et al., 1991; Romig et al., 1992), including topoisomerase II (Adachi et al., 1989; for a brief review, see Poljak et al., 1994).

EFFECTS OF MARS ON TRANSGENE EXPRESSION IN ANIMALS

Many experiments have been carried out to investigate the effects of MAR sequences on the expression of stably integrated transgenes in cells and organisms. In general, the conclusions drawn from these experiments have been that MARs increase overall levels of expression and decrease variability of expression. In some cases "position-independent" and "copy number-dependent" transgene expression have been claimed. It is not always clear, however, exactly what is meant by these terms. Transformant-to-transformant differences in transgene expression have traditionally been ascribed to "position effects." This usually means that the level of expression of a transgene is dependent on the chromatin structure (and the proximity of enhancers or silencers) in the region of the host genome into which the transgene becomes incorporated. Thus, in theory, "position independence" should indicate that all single-copy transformants (discounting deletions and rearrangements) would transcribe the transgene at essentially the same rate. In the absence of complicating phenomena, additional copies of the transgene should also be transcribed at the same rate, resulting in "copy-number dependence," i.e. more genes, more gene product. In practice, claims for MAR-mediated increases in transgene

expression have usually been convincingly demonstrated, but evidence for position independence and copy-number dependence has been less compelling. In fact, in absolute terms variability in expression of transgenes flanked by MARs is usually greater than controls, i.e. only when the higher mean values of MAR transformants are taken into account by using logarithmic transformations of the data or by using the coefficient of variation (SD divided by the mean) does the MAR-mediated decrease in variation become apparent.

The first work specifically designed to test the effect of MARs in transgene expression in animal cells in culture (chicken promacrophages) was that of Stief and co-workers (1989), who used the chicken lysozyme MAR flanking a reporter gene. In this work, average expression from the MAR transformants was about 10-fold greater than non-MAR controls. The variability of expression in low-copy-number MAR transformants was slightly less than the variability in controls (but only when logarithmically transformed data or the coefficient of variation were considered). Higher copy-number transformants were similarly variable in expression, but there was a general trend of higher gene expression at higher copy number, i.e. copy-number dependence. Phi-Van and co-workers (1990) carried out similar experiments with similar re sults. They claimed only "dampening" of position effects, but in this work as well, the 10-fold increase in average transgene expression was the most striking result.

Several other papers have appeared that generally corroborate the results of these two works. Bode and co-workers carried out extensive studies of the effects of MARs (Klehr et al., 1991). They used a variety of MARs, including some of plant origin, and consistently demonstrated MAR-mediated increases in transgene expression. They have not, however, claimed reduction in transformant-to-transformant variability in gene expression. Two recent papers may force a re-evaluation of our thoughts about the effects of MARs in transgene expression. Poljak and co-workers (1994) completed an extensive study of the effects of MARs on transgene expression in transfected mammalian cells in culture. They observed large MAR-mediated increases in average levels of gene expression but specifically denied copy-number dependency or reduction in variability. A direct comparison between these data and our earlier data from transformed tobacco cells in culture will be made in the section on the effects of MARs in plant systems. Kalos and Fournier (1995), also working with mammalian cells in culture, found MAR-mediated increases in transgene expression in low-copy transformants (one to two copies per cell), but in multicopy transformants transgene expression was strongly repressed. The results of these two studies with animal systems may indicate the involvement of "homology-dependent gene silencing," a well-recognized phenomenon in plants.

EFFECTS OF MARS ON TRANSGENE EXPRESSION IN PLANTS

The studies of the nuclear matrix, MARs, and the effects of MARs on transgene expression have been less extensive in plant systems than in animal systems. Until recently it

might have been accurate to say that the work with plant systems did not agree well with the animal work. But because the recent observations of Poljak and co-workers (1994) and Kalos and Fournier (1995) contrast so sharply with the early animal work, such a statement can no longer be made. The range of conflicting data with plant systems now simply fits into the overall range of conflicting data from all biological systems. As yet, we do not know the reasons for the disparate observations, but it is interesting to note that no two laboratories (working with plants or animals) have used the same combinations of MARs, reporter gene systems, cells (or whole organisms), or methods of transformation. We assume that further work will explain the apparently conflicting data. Here we will summarize very briefly the observations made with plant systems.

Results of an early plant study (Breyne et al., 1992) seem to disagree the most with other published data. Breyne et al. (1992) used tobacco cells in culture, *Agrobacterium*-mediated transformation, GUS as a reporter, and both a plant MAR and an animal MAR. They noted slight decreases in variability, but surprisingly overall levels of gene expression in MAR transformants were unaffected or slightly decreased. It is difficult to compare this work to others, however, because no gene copy numbers were determined. van der Geest and co-workers (1994) found that MARs flanking the bean β-phaseolin gene caused a modest increase in overall expression and modest decrease in variability in expression of a reporter gene in tobacco plants transformed using *Agrobacterium*. The work of Schöffl and co-workers (1993) comes the closest to what might have been expected based on the early work with animal systems. In this work a MAR isolated from a clone containing a soybean heat-shock gene was used to generate transgenic tobacco plants by *Agrobacterium*-mediated transformation. The MAR resulted in a 5- to 9-fold increase in expression of the GUS reporter gene, but no effect on the variability of expression was noted. There was, however, at least partial copy-number dependence of expression. Mlynárová and co-workers (1994, 1995) used an animal MAR (chick lysozyme) and a GUS reporter gene in *Agrobacterium*-mediated transformation of tobacco plants. They found a modest increase in average transgene expression (4-fold) and a substantial decrease in transformant-to-transformant variability in transgene expression (8-fold decrease in variance of logarithmically transformed data). In contrast to the plant work of Schöffl and co-workers (1993) but in agreement with the animal cell work of Poljak and co-workers (1994), no copy-number dependence in transgene expression was found.

In our work (Allen et al., 1993; Spiker et al., 1995) we have used both a heterologous (yeast) and a homologous (tobacco) MAR. We chose to introduce DNA into tobacco cells in culture using microprojectile bombardment, because this method of transformation allows straightforward co-transformation in which the selectable marker (NPTII for kanamycin resistance) and the reporter gene (GUS) are on different plasmids. The objective was to avoid the bias in favor of expression that may result from the close physical linkage of selectable marker and reporter genes in most *Agrobacterium* vectors. Another reason for using this approach is to facilitate comparison to the animal work, in which direct transformation using physically unlinked selection and reporter genes has been used in nearly all studies reported to date. Our results closely parallel those of Poljak and co-workers (1994). When we use a yeast MAR (a weak MAR by the exogenous matrix-binding assay), we see an overall stimulation in transgene expression of 12-fold (24-fold on a per-copy basis) (Allen et al., 1993). When a strong tobacco MAR is used, we see a greater than 60-fold stimulation (nearly 140-fold on a per-copy basis) (Spiker et al., 1995). In both of these cases, the variability of transgene expression is reduced only slightly, if at all, by the presence of MARs, and there is no evidence for copy-number dependence. In fact, transgene expression is inhibited at higher copy numbers. This inhibition is similar to that observed by Poljak and co-workers (1994) but not nearly so severe as the repression observed by Kalos and Fournier (1995) in multicopy transformants.

BY WHAT MECHANISM DO MARS AFFECT TRANSGENE EXPRESSION?

In the earliest work on the effect of MARs in transgene expression, it was established that MARs do not work as typical enhancers and must be incorporated into the host genome to have their effect (Stief et al., 1989). The observation that MARs have little effect in transient transcription assays has been consistent in both animal and plant systems (Klehr et al., 1991; Allen et al., 1993). Thus, because it appears as though chromatin structure is involved, the loop domain model of chromatin structure has influenced our thinking about how MARs might increase average levels of transgene expression by reducing position effects. One of the early models is illustrated in Figure 1. According to this model, when cells are transformed in the usual way, without using MARs, the introduced DNA is randomly incorporated into the host genome. If the DNA becomes incorporated into a domain of transcriptionally poised chromatin (like the domain on the left in Fig. 1A), the transgene will adopt the chromatin structure of that domain and thus have a high potential for transcription. Conversely, if the introduced DNA becomes incorporated into a transcriptionally inactive domain, like the one on the right, it will adopt an inactive chromatin structure and thus have a low potential for transcription. If MARs are used to flank the reporter gene construct, any introduced DNA that becomes incorporated into an inactive domain will form an independent domain insulated from the effects of the chromatin surrounding it. There is no a priori reason to predict that such an independent domain would assume a transcriptionally poised structure, as shown in Figure 1B. The formation of an active structure could, however, explain the high average levels of transgene expression when MARs are used to flank reporter gene constructs.

Although cloned MARs may allow transgenes to form independent loop domains, it is obvious that the model described above, at least in its simplest form, cannot completely explain the observed effects of MARs on transgene expres-

sion. If the model were literally interpreted, we would expect less variation in expression of low-copy transformants and much more consistent demonstration of copy number-dependent transgene expression. Several additional models have been suggested to explain the effects of MARs on transgene expression, such as that MARs form nucleation points for DNA unwinding (Bode et al., 1992), that MARs form sites of nucleation for HMG proteins to displace H1 histones (proteins involved in highly coiled chromatin fibers) (Käs et al., 1993), and that MAR elements stabilize chromosomal topology arising as a consequence of hyperacetylation of histone cores (Schlake et al., 1994).

We have hypothesized that the model depicted in Figure 1B is essentially applicable but that complicating phenomena, including homology-dependent gene silencing, are also involved (Allen et al., 1993). According to our model, MARs can stimulate transgene expression by reducing the severity of homology-dependent gene silencing. (For a recent review of gene silencing see Matzke and Matzke, 1995.) The observation that MARs have smaller effects when used in *Agrobacterium*-mediated transformation than in direct DNA-mediated co-transformation can be rationalized in part by noting that direct transformation normally leads to complex multicopy arrays of transgenes at a single locus and that such configurations are more likely to provoke gene silencing than the generally simpler integration events mediated by T-DNA (Matzke and Matzke, 1995). In addition, the linkage of selectable and reporter genes in T-DNA probably excludes from analysis many transformants in which gene silencing leads to low expression of the entire T-DNA locus. Such an exclusion would result in increasing the average expression among control transformants and reducing the apparent magnitude of any additional MAR effect. In addition, we note that most of the MAR-mediated reduction in variability observed by Mlynárová and co-workers (1994, 1995) seems to have arisen from reducing the number of low expressors in their transformant populations. Thus, one possible interpretation of their results is that MARs reduce reporter gene silencing even more effectively than does selection for a linked resistance marker.

As mentioned in the opening paragraph, work with MAR sequences is still in its early stages, especially in plant systems. Much remains to be learned about how MARs interact with the nuclear matrix, what role MARs have in organizing DNA in the nucleus, and by what mechanism MARs affect transgene expression. However, we believe that MARs may find practical application even before the mechanistic basis for their effects is understood. It seems likely that MARs will soon be widely used as boosters of transgene expression or to combat gene silencing, especially in situations in which the transforming DNA must be introduced directly rather than with *Agrobacterium*. It is tempting to speculate further that MARs may also reduce the incidence of gene silencing in advanced generations of transgenic lines, a problem with serious practical implications (Finnegan and McElroy, 1994). That such poorly understood sequence elements should have such great potential utility emphasizes the depth of our ignorance about gene regulation at the chromatin level.

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