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Use of matrix attachment regions (MARs) to minimize transgene silencing

George C. Allen[1,*], Steven Spiker[2] and William F. Thompson[1,2,3]
[1]*Department of Botany, 2214 Gardner Hall, Box 7612,* [2]*Department of Genetics, and* [3]*Department of Crop Science, North Carolina State University, Raleigh, NC 27695, USA (*[*]*author for correspondence; e-mail george_allen@ncsu.edu)*

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Abstract

Matrix attachment regions (MARs) are operationally defined as DNA elements that bind specifically to the nuclear matrix *in vitro*. It is possible, although unproven, that they also mediate binding of chromatin to the nuclear matrix *in vivo* and alter the topology of the genome in interphase nuclei. When MARs are positioned on either side of a transgene their presence usually results in higher and more stable expression in transgenic plants or cell lines, most likely by minimizing gene silencing. Our review explores current data and presents several plausible models to explain MAR effects on transgene expression.

Abbreviations: MAR, matrix attachment region; LBAR, loop basement-associated region; HMG, high-mobility group; BUR, base unpairing region; Gus, β-glucuronidase; BC-1, back-cross-1; H1, histone H1; MATH, multiple AT hook; PEV, position effect variegation; GFP, green fluorescent protein

Introduction

Gene transfer technology is being used to enhance agronomic performance or improve quality traits in a wide variety of crop species, and has become a fundamental tool for basic research in many subdisciplines of plant biology. However, both practical applications and basic research are sometimes severely handicapped by difficulty in obtaining material in which transgene expression is predictable and stable over many generations.

Such problems were first encountered as variation among independent primary transformants (or 'transgenic events') and were thought to arise largely from position effects similar to those that had previously been documented in *Drosophila* (Weiler and Wakimoto, 1995). It was thought that, because integration seemed to occur randomly in the plant genome, some transgenes would be integrated in a relatively uncondensed, transcriptionally active chromatin environment, while others would be integrated into regions of the genome characterized by a condensed, transcriptionally inert chromatin structure. More recently, we have come to appreciate that factors other than chromatin structure can also contribute to expression variability. Prominent among these factors is homology-dependent gene silencing, the subject of this special issue of Plant Molecular Biology.

Our laboratories have been investigating the relationship between nuclear structure and gene expression, looking for DNA sequences that could be included in transgene constructs to increase the reliability of transgene expression. We have recently been focusing on sequences that mediate binding of chromatin to the nuclear matrix, a network of proteinaceous fibers that permeates the nucleus and presumably functions to organize chromatin into a series of topologically isolated loop domains. We believe that such sequences – known as matrix attachment regions, or MARs[1] – may influence the structure of transgene

[1]The DNA sequences we call MARs (Cockerill and Garrard, 1986) are also called SARs (scaffold attachment regions (Gasser and Laemmli, 1986b; Mirkovitch *et al*., 1986). Both terms apparently describe the same biological entity (Bode and Maas, 1988). We use

chromatin and/or its ability to interact with other sequences in the nucleus. Data from several laboratories indicate that MARs enhance transgene expression to varying extents in different systems and are consistent with the hypothesis that MARs can reduce or eliminate some forms of gene silencing.

Structure and composition of the nuclear matrix

Much of what is currently known about the nuclear matrix comes from studies in animal systems. Only recently has there been much interest in the plant nuclear matrix (Hall *et al*., 1991; Paul and Ferl, 1998; Gindullis and Meier, 1999; Hatton and Gray, 1999), and relatively little structural information is yet available. However, it has been reported that the protein composition of animal and plant matrices is highly conserved (Moreno Diaz de la Espina, 1995). Thus, we will rely heavily on the animal literature for information on nuclear structure.

The nuclear matrix was originally described by Berezney and Coffey (1974). It is visualized as a network of proteinaceous fibrils within the nucleus, and is thought to interact with cytoskeletal elements in the cytoplasm (Wan *et al*., 1999). In intact nuclei, chromatin fibers are thought to attach to the matrix at frequent intervals. Biochemically, matrix preparations are isolated by selectively extracting nuclei so as to remove the majority of histones and DNA. The remaining complex, composed mostly of proteins and structural RNAs (Verheijen *et al*., 1988; Spector, 1990) is operationally defined as the nuclear matrix. Early procedures involved a combination of high-salt extraction and digestion with DNase I (Berezney, 1984; Cockerill and Garrard, 1986). More recently, most authors have used low concentrations of chaotropic agents such as LIS (lithium diiodosalicylate) to achieve selective extraction under conditions in which precipitation artifacts are minimized (Mirkovitch *et al*., 1984; Hall *et al*., 1991; Hall and Spiker, 1994) and have substituted restriction enzymes for DNase I. The result is a proteinaceous network (Figure 1) associated with a residual fraction of DNA fragments. This residual fraction has been shown to be greatly enriched for MAR-containing DNA fragments[2] .

Chromatin structure and gene transcription

For a gene to be expressed, RNA polymerase and the associated transcription factors must have access to the DNA binding sites (Jackson, 1997). The availability of the DNA binding sites is a function of the local chromatin structure and can be considered to be analogous to a coarse control. Once the DNA binding sites become available, finer control can be exerted by regulating the abundance or activity of gene-specific transcription factors (Bode and Maas, 1988; Bodnar, 1988).

Experimentally, the most common way of measuring the accessibility of chromatin surrounding a gene is to determine its sensitivity to digestion when isolated nuclei are treated with DNase I (Weintraub and Groudine, 1976). General DNase I sensitivity typically extends tens of kilobases beyond the gene itself into the flanking regions. Changes in general DNase I sensitivity therefore reflect changes in higher-order chromatin structure within a region of the genome.[3]

The basis for general DNase I sensitivity is unknown. Cytologically characterized 'heterochromatin' and 'euchromatin' do not correspond to DNase I-resistant and -sensitive chromatin, as some euchromatin is DNase I-resistant (Olszewska, 1992). It is tempting to associate DNase I resistance to 30 nm chromatin fibers and DNase I sensitivity with 11 nm nucleosome fibers. However, 11 nm fibers probably do not exist *in vivo*, and differences in DNase I sensitivity are more likely to reflect differences in the degree of compaction of the so-called 30 nm fiber (Van Holde and Zlatanova, 1995).

Where regions of general DNase I sensitivity have been mapped, they have been shown to extend over large distances, and frequently include several adjacent genes (Stalder *et al*., 1980). Thus, eukaryotic genomes appear to contain defined regions, or *domains*, of chromatin that are organized in a relatively open conformation (Stalder *et al*., 1980; Levy-Wilson and Fortier, 1989; Bonifer *et al*., 1991). This conformation is sometimes referred to as 'transcriptionally poised' because it creates the potential for transcription to occur whenever the requisite transcription factors and accessory proteins are present and active.

the term 'MAR' because of its prevalence in the literature, and we avoid the hybrid nomenclature of S/MAR (Bode *et al*., 1995).

[2]DNA in this fraction is sometimes assumed to be matrix-associated *in vivo*. This assumption should be viewed with caution, however, because DNA that has the potential to bind to the nuclear matrix but is not actually bound to it *in vivo* may become associated with the matrix at any point during the extraction and digestion procedure.

[3]General DNase I sensitivity can be distinguished from DNase I hypersensitivity in that DNase I-hypersensitive sites are limited to a few hundred base pairs and are generally considered to be sites devoid of nucleosomes (Elgin, 1988).

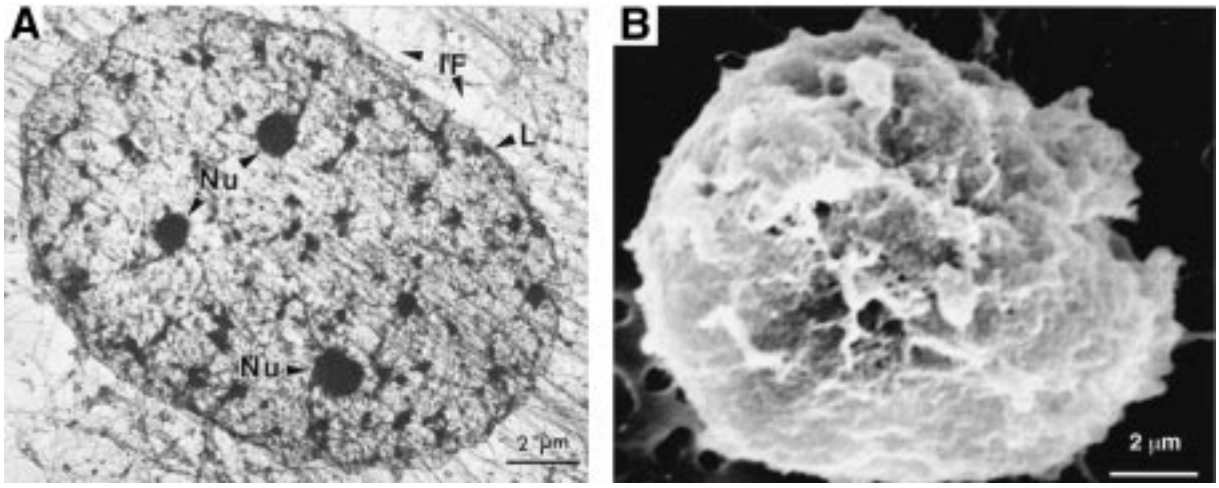

Figure 1. A. Transmission electron micrograph of amine-modified nuclear matrix from Wan *et al.* (1999). Animal cell nuclei were extracted and digested with restriction enzymes. Nuclear matrices are bounded by a nuclear lamina (L), and enmeshed in and connected to the intermediate filaments (IF) of the cytoskeleton. The nucleoli (Nu) are also connected to nuclear matrix filaments. Figure reproduced from Figure 3 of Wan *et al.* (1999) by permission of the National Academy of Sciences. B. Scanning electron micrograph of tobacco nuclear matrix from cultured NT-1 cells. Nuclei were extracted with LIS and digested with restriction enzymes. The micrograph was kindly provided by Tom Phelan and Tuyen Nguyen, North Carolina State University, USA.

Having the potential to be transcribed distinguishes these regions from the vast majority of the genome, in which varying degrees of higher-order structure prevent access to the DNA.

Loop domain model

When the borders of DNase I-sensitive domains were cloned and characterized, it was discovered that MARs tended to occur in these regions (Bode and Maas, 1988; Levy-Wilson and Fortier, 1989; Bonifer *et al*., 1991). This result is consistent with a 'loop domain' model, in which chromatin fibers are thought to be organized into a series of loops attached at their bases to the nuclear matrix. Loop domains have also been visualized in electron micrographs of histone-depleted mitotic chromosomes (Paulson and Laemmli, 1977), and it is usually assumed that the domains defined by DNase I sensitivity experiments correspond to a subset of the loops seen in electron micrographs. Each of the loops is thought to form a topologically isolated unit in which processes such as chromatin condensation and DNA replication can be regulated independently (Marsden and Laemmli, 1979; Bodnar, 1988; Hassan *et al*., 1994). It is thought that some loops remain permanently condensed and inactive, even in euchromatic portions of the genome, while others can be extended to produce a transcriptionally poised conformation in appropriately differentiated cells.

Loop domains in *Drosophila* have been estimated to range from 4.5 kb to hundreds of kilobases in size, based on experiments in which the positions of MARs were mapped in large cloned regions of the genome (Gasser and Laemmli, 1986a; Mirkovitch *et al*., 1986). In HeLa cells, biochemical data are consistent with a genome-wide average loop size of 86 kb, with most loops falling in a range of 70 to 100 kb (Jackson *et al*., 1990a, b). Plant nuclei also appear to contain a wide range of loop sizes, although the average size may be smaller than it is in animals. For example, Avramova *et al*. (1995) examined MAR binding within a contiguous 280 kb region from maize chromosome 1, finding nine potential loop domains ranging from 6 kb to greater than 75 kb and averaging ca. 30 kb. The *adh1* gene, which has frequently been used as a model for chromatin structure studies, was found to reside in either a 3 kb or a 12 kb loop, depending on whether a strong or weak MAR was assumed to define the 3′ end of the domain. In tobacco, Michalowski *et al*. (1999) used *in vitro* binding data to estimate the number of MAR binding sites for the tobacco Rb7 MAR, and calculated an average loop size of 17 kb. All these loop size estimates assume that sequences defined as MARs by *in vitro* binding assays actually form the bases of loops *in vivo*. This assumption ignores the strong possibility that not all MARs have equivalent functions.

For example, Paul and Ferl (1998) have proposed a model for plant genome organization in which relatively large loop domains are created by a subset of MARs known as 'loop basement attachment regions' (LBARs). Other MARs might exist within the LBAR domains, and different types of MARs might be envisioned to have different functions in gene expression and chromosome organization.

Recent genome sequencing efforts of collinear regions of maize, sorghum, and rice have shown that even in the absence of sequence homology, the locations of MARs tend to be preserved (Avramova *et al*., 1998; Tikhonov *et al*., 2000). Such results suggest that during evolution, while sequence divergence occurs, higher-order nuclear structure is conserved.

In evaluating the loop domain model, it is important to remember that our understanding of the nuclear matrix depends critically on the analytical techniques employed to analyze it. For example, there has been much discussion on the extent to which histone extraction, which is necessary to unpack the chromatin, may alter the structure of the matrix (Jackson and Cook, 1985; Mirkovitch *et al*., 1987). Indeed, the very existence of the nuclear matrix as an *in vivo* structure has been questioned (Cook and Jackson, 1988). However, the rapidly accumulating evidence for a very high degree of organization within the nucleus is consistent with the presence of some kind of structural framework. In addition, several lines of evidence link structural organization to gene expression in a way that is consistent with the loop domain model. For example, Lawrence's group used high-resolution fluorescence microscopy to view nuclear 'halo' structures, which result from expansion of the DNA upon selective removal of the histones (Gerdes *et al*., 1994). Pulse labeling with bromodeoxyuridine was used to demonstrate that the nascent replicating DNA was localized to the base of the chromatin loops, near the 'residual nucleus' or matrix. Increasing the duration of the pulse resulted in signals that were progressively further and further away from the matrix. Such a result is consistent with a hypothesis that replication is initiated at the nuclear matrix, and that the relevant associations between chromosomal DNA and the nuclear matrix persist after histone extraction. Croft *et al*. (1999) showed that human chromosome 19 occupies a more internal position in the interphase nucleus than chromosome 18, and is also more extensively associated with the nuclear matrix. An earlier analysis of replication timing showed that chromosome 19 also replicates earlier in the cell cycle than chromosome 18 (Dutrillaux *et al*., 1976), a result that is consistent with a model in which MARs are important elements in DNA replication (Ma *et al*., 1998; Cook, 1999).

Gerdes *et al*. (1994) also used *in situ* hybridization to localize several genes within the nuclear halos, and to compare the locations of transcriptionally active and inactive genes. Signals obtained with probes for inactive genes were frequently found in the extruded DNA surrounding the nuclear matrix. However, active genes were observed as discrete spots of hybridization associated with the matrix. These differences in localization were apparent only in nuclear halos; prior to histone extraction there were no obvious differences between active and inactive loci. Such data imply that transcriptionally active genes are more closely associated with nuclear matrix than transcriptionally inactive genes, and are consistent with earlier proposals (Gasser and Laemmli, 1987) that genes in close proximity to the nuclear matrix are more likely to be expressed.

Abranches and Shaw (manuscript in preparation) studied the location of the transgenic loci in nuclei from some of the tobacco plants originally produced by Ülker *et al*. (1999). The plants, which had been transformed by bombardment with 35S::Gus constructs with or without flanking Rb7 MARs, were selected to contain similar numbers of transgenes. Abranches and Shaw extracted the histones from the nuclei to produce nuclear halos, using a procedure similar to that of Gerdes *et al*. (1994), and then used fluorescence *in situ* hybridization to locate the transgenes. The micrographs in Figure 2 show that expressed, MAR-containing transgenic loci are located close to the residual matrix, while control loci without MARs are more likely to be found in the halo of DNA surrounding the matrix. This *in situ* procedure still does not actually measure matrix association *in vivo*, but artifactual associations between MARs and the matrix may be less likely when histones are extracted *in situ* and DNA is not digested than they are in standard matrix isolation procedures. Thus the results support the hypothesis that MAR-flanked transgenes are more closely associated with the nuclear matrix *in vivo*.

MAR effects on transgene expression

Much of the early work on MAR effects was based on the supposition that most of the variation seen in transgene expression resulted from the randomness of the

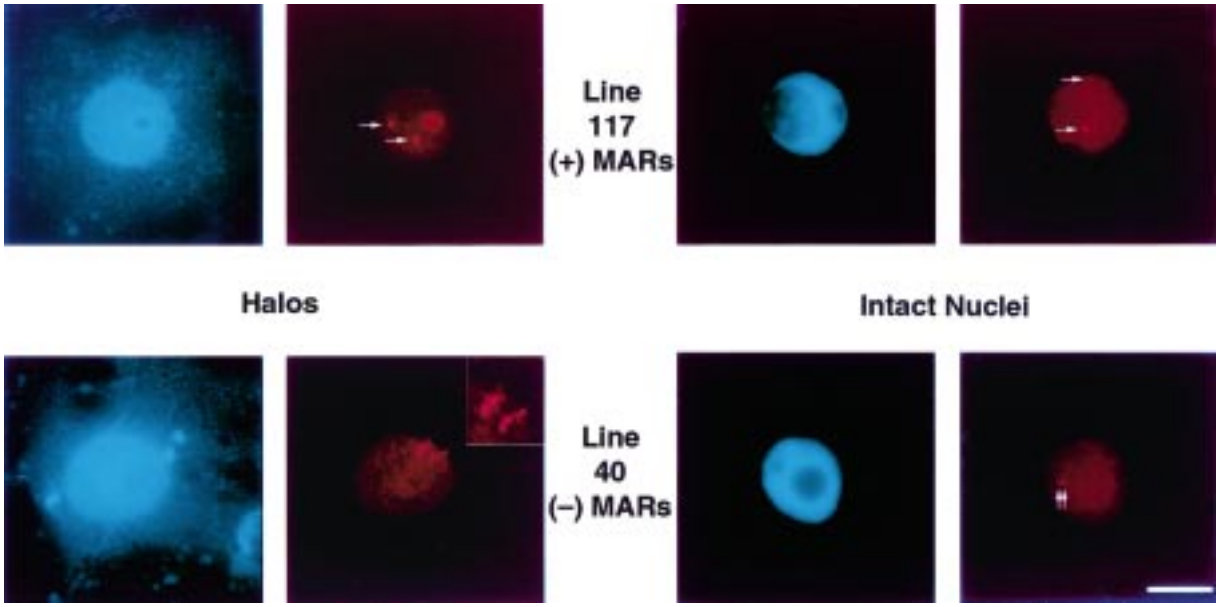

Figure 2. Visualization of transgene loci by fluorescence *in situ* hybridization to histone-depleted nuclear halos or intact nuclei. Micrographs were kindly provided by Rita Abranches and Peter Shaw, John Innes Institute, UK. Top row: *in situ* hybridization to nuclear halos (left) and intact nuclei from plants transformed with a MAR-flanked 35S::Gus transgene. The MAR plant (a doubled haploid of Line117 from Ülker *et al*., 1999) contained six copies of the transgene. Bottom row: *in situ* hybridization to nuclear halos (left) and intact nuclei from plants transformed with a control 35S::Gus transgene lacking MARs. The plant (a doubled haploid of Line 40 from Ülker *et al*., 1999) contained seven copies of the transgene.

insertion process. It was frequently assumed that transgene integration into, or near, condensed chromatin would give rise to reduced and/or variable expression similar to the well-documented phenomenon of 'position effect variegation' in *Drosophila* (Weiler and Wakimoto, 1995; Sabl and Henikoff, 1996). Expression variation could also be generated by integration at variable positions with respect to endogenous regulatory elements such as enhancers. Support for the latter idea comes from the high frequency with which 'enhancer trap' constructs are expressed in plant cells (Beilmann *et al*., 1992; Sundaresan *et al*., 1995; Campisi *et al*., 1999).

In such cases, the loop domain model suggests that MARs might act as boundary elements to reduce the influence of adjacent regulatory elements or prevent the spread of chromatin condensation into a transgene domain. If the role of MARs were simply to protect a transgene from endogenous influences, one would predict that total expression from several copies of the same transgene would be simply related to the number of copies inserted. Indeed, early reports with animal cells showed that MAR-flanked transgenes did express at levels proportional to copy number (Stief *et al*., 1989). As will be apparent below, however, subsequent work from many laboratories has shown that the overall picture is more complex (Bonifer *et al*., 1994; Huber *et al*., 1994; Phi-Van and Strätling, 1996).

Table 1 summarizes the current data concerning MAR effects on transgene expression in plants and plant cells in culture. Most reports agree that MARs cause moderate increases in expression in plants transformed with *Agrobacterium* vectors (Schöffl *et al*., 1993; van der Geest *et al*., 1994; Mlynárová *et al*., 1994, 1995; Han *et al*., 1997; Liu and Tabe, 1998; Levee *et al*., 1999). Using biolistics to stably transform tobacco cells in culture, however, we observed an average 12-fold increase with a MAR from yeast, and a 60-fold increase with a MAR from tobacco that binds more strongly to the nuclear matrix (Allen *et al*., 1993, 1996). Because the selectable and reporter genes were not physically linked in the biolistic experiments, we considered the possibility that the close linkage between the selection and reporter genes in T-DNA vectors might limit the magnitude of the MAR effect. Experiments in animal systems have demonstrated that the MAR effect is minimized by such linkage (Mielke *et al*., 1990), presumably because less productive integration events are selected against. Interestingly, however, we have recently shown that the

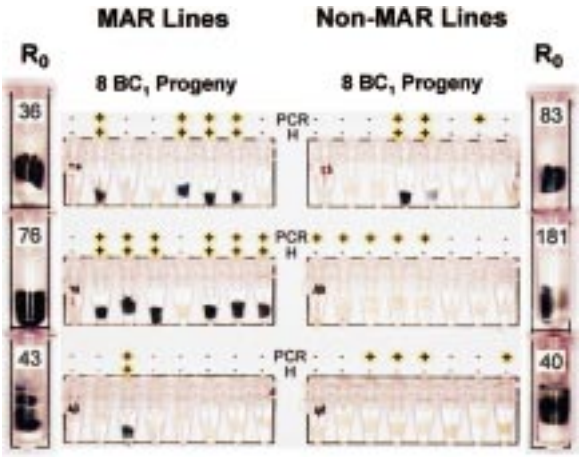

Figure 3. Examples of GUS histochemical staining and PCR analysis for selected BC-1 lines from a population of tobacco plants transformed with constructs containing or lacking MARs. The GUS histochemical staining results for the R_0 plants are compared with the results obtained from their BC-1 progeny. Gus expression is indicated by blue staining in the leaf punches shown. H indicates histochemical staining; + indicates Gus expression detectable by this assay and − indicates the absence of detectable Gus expression. PCR indicates a polymerase chain reaction result, + the presence and − the absence of an amplified junction fragment. Reproduced from Ulker *et al.* (1999) by permission of Plant Journal.

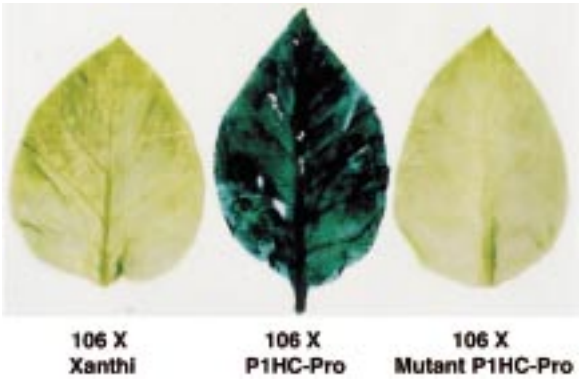

Figure 4. Reactivation of expression by a viral anti-silencer in a post-transcriptionally silenced tobacco line containing transgenes flanked by MARs. The figure shows GUS staining in leaves of progeny tobacco plants from crosses between parental plants homozygous for the MAR-containing transgene locus in line 106 (Ulker *et al.*, 1999) and either plants homozygous for the post-transcriptional suppressor P1HC-Pro (center) or plants containing a non-translatable mutant of P1HC-Pro (right). The leaf on the left represents progeny of a cross between untransformed Xanthi control plant and the homozygous line 106 parent. The photo was kindly provided by Vicki Vance and Allison Mallory, University of South Carolina, USA.

same biolistic procedure and the same strongly binding MAR that increased average expression by 60-fold in cultured cells produced only a 2- to 3-fold effect in regenerated tobacco plants (Ülker *et al.*, 1999). Vain *et al.* (1999) have shown a similarly moderate effect in regenerated rice plants subjected to biolistic co-transformation.

The fact that we see a lower MAR effect in more quiescent, slowly dividing cells of regenerated plants has led us to suggest that differences in the type of plant tissue can lead to differences in the MAR effect. Precedent for this suggestion exists in animal systems, where Thompson *et al.* (1994) have noted a large MAR effect on transgene expression in embryonic mouse cells, but a much smaller effect in the differentiated cells of adult tissues. As will be discussed further below, it may be significant that HMG I/Y protein levels are also known to be higher in rapidly dividing, undifferentiated cells (Johnson *et al.*, 1990), because HMG I/Y proteins have been proposed to interact with MARs to create an open chromatin structure (Käs *et al.*, 1993).

Characteristics of MAR elements

We define MARs operationally by two criteria: (1) as endogenous DNA fragments that copurify with the nuclear matrix, remaining bound while most of the DNA is removed by endonuclease digestion, and (2) as exogenously added DNA fragments that bind to the purified nuclear matrix in the presence of competitor DNA (Hall *et al.*, 1991; Hall and Spiker, 1994).

Nearly all characterized MARs are AT-rich (greater than 65%) (Boulikas, 1995), and MARs generally contain regions that tend to produce single-stranded or base unpairing regions (BURs) that can be identified using chemical probes that react with unpaired DNA bases (Kohwi-Shigematsu and Kohwi, 1990). Benham *et al.* (1997) have shown that the organization of the BUR motifs to be important MAR identifier. Thus, the affinity of a MAR for the nuclear matrix appears to be affected by the location and organization of the AT-rich regions. However, simply being AT-rich does not make a DNA fragment a MAR (Mielke *et al.*, 1990). Several MAR motifs have been suggested (Boulikas, 1995; van Drunen *et al.*, 1997) but these have been of minimal use in predicting the affinity of a DNA fragment for the nuclear matrix. We have approached the problem by shotgun cloning MAR elements from tobacco cells and comparing the

Table 1. Effect of flanking MARs on transgene expression and variability in transformed plants and plant cells.

Plant system	Source of MAR	Promoter-reporter	DNA transfer	Effect on expression level	Effect on expression variability	Reference
Tobacco cells	Soybean	nos-GUS	T-DNA	Small decrease	Small decrease	(Breyne *et al.*, 1992)
	Human	nos-GUS	T-DNA	No effect	Small decrease	
Tobacco plants	Soybean	Heat Shock-GUS	T-DNA	9-fold increase	No effect	(Schöffl *et al.*, 1993)
Tobacco cells	Yeast	35S-GUS	Biolistic	12-fold increase	Small	(Allen *et al.*, 1993)
Tobacco plants	Chicken	Lhca3-GUS	T-DNA	4-fold increase	3-fold decrease	(Mlynárová *et al.*, 1994)
	Chicken	Lhca3-GUS	T-DNA	3-fold increase	7-fold decrease	
Tobacco plants	Bean	Phaseolin-GUS	T-DNA	3-fold increase	2-fold decrease	(van der Geest *et al.*, 1994)
Tobacco plants	Chicken	Enh35S-GUS	T-DNA	2-fold increase	2-fold decrease	(Mlynárová *et al.*, 1995)
	Chicken	Enh35S-GUS	T-DNA	2-fold increase	7-fold decrease	
Tobacco cells	Tobacco	35S-GUS	Biolistic	60-fold increase	No effect	(Allen *et al.*, 1996)
Poplar explants	Tobacco	35S-GUS	T-DNA	10-fold increase	Small	(Han *et al.*, 1997)
Tobacco leaf discs	Tobacco	35S-GUS	T-DNA	Small	Small	
Tobacco plants	*Arabidopsis*[4]	35S-GUS	T-DNA	5- to 10-fold increase	No effect	(Liu and Tabe, 1998)
Maize cells	Chicken	35S- cabl-GUS	Biolistic	36-fold increase	Increase	(Odell and Krebbers, 1998)
Rice plants	Tobacco	35S-GUS	Biolistic	2.5-fold increase	Small	(Vain *et al.*, 1999)
	Yeast	35S-GUS	Biolistic	3-fold increase	Small	
Tobacco plants	Tobacco	35S-GUS	Biolistic	2-fold increase	Small	(Ülker *et al.*, 1999)
Pine callus	Tobacco	Enh35S-GUS:nptII	T-DNA	3 to 4-fold	Decrease	(Levee *et al.*, 1999)
Sorghum cells	Tobacco	Ubiquitin-GUS	Biolistic	2-fold increase	No effect	(Able *et al.*, 2000)

sequences of fragments with varying affinities for the nuclear matrix (Michalowski *et al*., 1999). This procedure allowed us to identify a new motif that we call the '90% AT box' (a sequence of 20 bp of which 90% or greater are AT). The frequency of this motif more highly correlated with binding strength than the frequencies of any previously described MAR motifs.

Table 1 summarizes the variety of MARs that have been tested in plants. In the following two sections we will highlight two particular elements that have been extensively characterized and used in a variety of plant transformation studies.

The A element and copy number dependence

The chicken lysozyme locus contains a ca. 3 kb regulatory region known as the A element. This element was originally used as a MAR in a series of experiments on the effect of MARs on gene expression (Stief *et al*., 1989; Bonifer *et al*., 1990; Phi-Van *et al*., 1990). These experiments were exciting because transgenes flanked by the A element exhibited expression that was proportional to gene copy number ('copy number-dependent'), suggesting that the element had been able to insulate transgene expression from gene silencing or position effects. Recently, however, more detailed studies have shown A element effects to be more complicated than originally thought (Bonifer *et al*., 1994; Huber *et al*., 1994; Phi-Van and Strätling, 1996). The intact element has been shown to contain both enhancer and matrix-binding activities (Bonifer *et al*., 1994). When the intact element was divided into 1.32 and 1.45 kb pieces, both were able to confer copy number-dependent transgene expression. However, when smaller fragments were tested, the portion of the A element that bound to the nuclear matrix no longer conferred copy number dependence (Phi-Van and Strätling, 1996), and the possibility must be considered that at least some of the original effects were attributable to the enhancer portion of the element rather than the matrix-binding portion.

Only the intact 3 kb A element has thus far been tested in plants. Interestingly, data from Nap's group on tobacco plants transformed with T-DNA vectors show that the A element can lead to modest increases in reporter gene expression and dramatic reductions in the variation among independent transformants as shown in Table 1 (Mlynárová *et al*., 1994, 1995, 1996). It would be informative to determine whether one or both of these effects are lost when only the minimal matrix binding fragment is used, as the animal data make it seem likely that other portions of the A element may be responsible for the reduced variation.

Given the complexity of the A element, Poljak *et al*. (1994) carried out experiments with several 'minimal MARs' that were carefully selected to contain only regions with matrix-binding activity. Testing these MARs in *Drosophila* tissue culture cells, they observed large increases in expression but no effect on the variation from transformant to transformant. Their results stand in contrast to those in which a minimal matrix-binding fragment of the A element had no effect on expression. Clearly, we need to know much more about different MARs and their binding sites before we can begin to understand such differences.

The Rb7 MAR

The Rb7 MAR is a 1.2 kb tobacco DNA fragment isolated from a region 3′ to the root-specific gene Rb7 (Conkling *et al*., 1990; Hall *et al*., 1991). It has been used in a variety of plant transformation systems, including cultured tobacco cells (Allen *et al*., 1996), tobacco plants (Ülker *et al*., 1999), rice (Vain *et al*., 1999), poplar explants (Han *et al*., 1997), sorghum cells (Able *et al*., 2000), and pine callus (Levee *et al*., 1999). The RB7 MAR is very AT-rich (73.2%) and has a high affinity for isolated tobacco nuclear matrices (Michalowski *et al*., 1999). We have estimated the number of Rb7 MAR-binding sites as ca. 400 000 per nucleus (Michalowski *et al*., 1999). Several restriction fragments within the 1.2 kb Rb7 MAR have also been tested for binding to the isolated tobacco nuclear matrix and it has been found that a 750 bp region contains the majority of the matrix-binding DNA (Hall, unpublished). It remains to be determined whether this 750 bp region can confer all of the effects seen with the original element.

Do MARS affect gene silencing?

The original hypothesis that MARs protect against homology-dependent gene silencing came from our work using the yeast MAR flanking the 35S::Gus reporter cassette (Allen *et al*., 1993). The goal of that study was to determine if MARs flanking a transgene could protect against position effects and thus give expression proportional to gene copy number or 'copy number-dependent expression' similar to what had been observed with the A element in an animal system. We did not find copy number-dependent expression in

our plant cell system, but we did find that a MAR from yeast and the Rb7 MAR greatly increased the mean expression level in a population of transformants. We also found that increasing the transgene copy number above a threshold level actually decreased expression, but that the threshold level seemed to be higher in populations transformed with MAR constructs. The observation that gene expression decreased above a 'threshold' copy number, and the fact that expression levels were very low in our control transformants, led us to suggest that homology-dependent gene silencing might be involved, rather than classical position effects (which are mediated solely by pre-existing features of the integration locus). Therefore, we speculated that MARs might resist gene silencing (Allen *et al*., 1993, 1996). This hypothesis was consistent with reports from the Signer and Henikoff laboratories indicating that even short arrays of repeated transgenes are subject to transcriptional silencing in *Arabidopsis* and *Drosophila*, respectively (Assaad *et al*., 1993; Dorer and Henikoff, 1994).

In a subsequent study, we (Ülker *et al*., 1999) showed that MARs reduced the loss of reporter gene expression from one generation to the next. Expressing R_0 plants were backcrossed to wild type, and BC-1 progeny that inherited the reporter gene were examined for expression using a GUS histochemical assay. As shown in Figure 3, most of the non-MAR plants gave rise to progeny in which the Gus gene is not expressed. In contrast, most of the plants transformed with the MAR-flanked reporter gene give rise to progeny that do express the reporter gene. DNA gel blots showed the same banding pattern for R_0 and BC-1 plants, indicating that loss of expression almost certainly reflected gene silencing rather than deletion or rearrangement of the transgene locus. These results support the hypothesis that MARs reduce the frequency of gene silencing events, especially during sexual reproduction.

Because transgenic loci produced by bombardment often contain arrays of sequences repeated *in cis*, it is likely that the silencing we observed was induced by *cis* interactions among the transgenes at a single locus. It remained to be determined whether or not MARs could protect against *trans* silencing mediated by sequence homologies elsewhere in the genome. To test this possibility, the Vaucheret lab (1998) crossed their 'supersilencer' tobacco line V271 (Vaucheret, 1993) with tobacco plants containing various MAR-flanked reporter gene constructs. The V271 line carries a complex multicopy locus derived from an antisense nitrate reductase construct driven by the 35S promoter (35S::RiN-NosT). This locus has been shown to silence transcription of 35S-driven promoters at a variety of different locations in the genome. V271 was crossed with plants produced in several of the studies shown in Table 1 (Mlynárová *et al*., 1994; van der Geest *et al*., 1994; Ülker *et al*., 1999). Hybrid plants carrying both the V271 and MAR-flanked 35S::Gus reporter loci were tested for GUS activity. In all cases, the MARs did not prevent *trans* silencing by the V271 locus. In a parallel set of experiments Vaucheret *et al*. (1998) also crossed the various MAR-containing plants with a plant known to cause highly efficient post-transcriptional silencing of Gus (plant 6b8) (Elmayan and Vaucheret, 1996). GUS expression was not observed in progeny from these crosses, indicating that MARs also do not protect against post-transcriptional silencing. Taken together, these results suggest that MARs are effective mainly against transcriptional silencing that is induced by *cis* interactions within repeated transgene arrays.

We now have evidence that when silencing occurs in plant lines carrying MAR-flanked transgenes, it is primarily at the post-transcriptional level. We have tested plants produced from the Ülker *et al*. study (1999) in crosses with plants expressing a viral suppressor of post-transcriptional silencing, P1HC-Pro (Anandalakshmi *et al*., 1998). When plants carrying a silenced Gus transgene flanked by MARs are crossed with lines expressing P1HC-Pro, expression is reactivated in the progeny (Figure 4). In contrast, Gus transgenes not flanked by MARs are not reactivated by a P1HC-Pro-expressing plant (Ascenzi *et al*., in preparation). Because P1HC-Pro is known to specifically suppress post-transcriptional silencing (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Kasschau and Carrington, 1998), these data are consistent with the hypothesis that MARs protect against transcriptional silencing, but that this protection becomes irrelevant if post-transcriptional silencing occurs. Because one way post-transcriptional silencing is thought to be induced is by accumulation of transcripts above a threshold level (Que *et al*. 1997 and reviewed in Gallie, 1998; Stam *et al*., 1997), it is possible that post-transcriptional silencing accounts for most of the decline in expression we observed in cell lines or plants containing high copy numbers of MAR constructs (Allen *et al*., 1993, 1996). This possibility is currently being investigated.

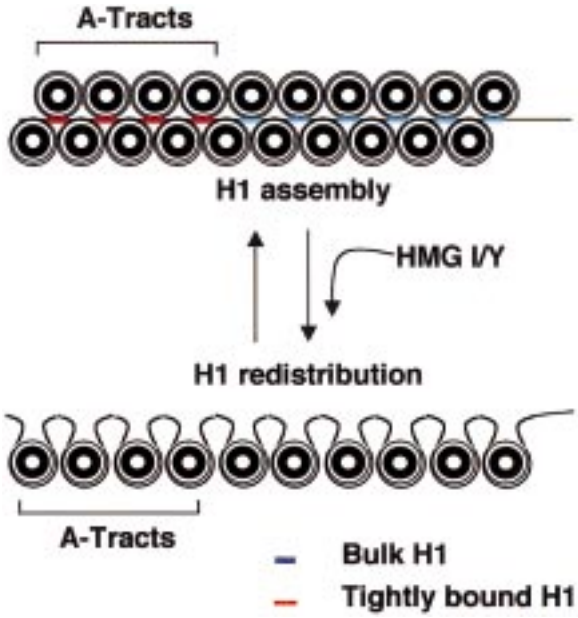

Figure 5. Chromatin opening model (adapted from Käs *et al.*, 1993). The top portion of the figure represents nucleosomes with tightly bound histone H1 molecules (shown as red or blue bars). Histones bound to A-tracts, which are thought to constitute MARs, are tightly bound by the H1 molecules shown as red bars. These H1 histones are thought to nucleate cooperative assembly of additional H1 histones (represented by the blue bars) onto adjacent nucleosomes, causing condensation of the chromatin. In the presence of HMGI/Y, however, H1 histone is displaced from the A tracts, which results in redistribution of bulk H1 histone and opening of the chromatin.

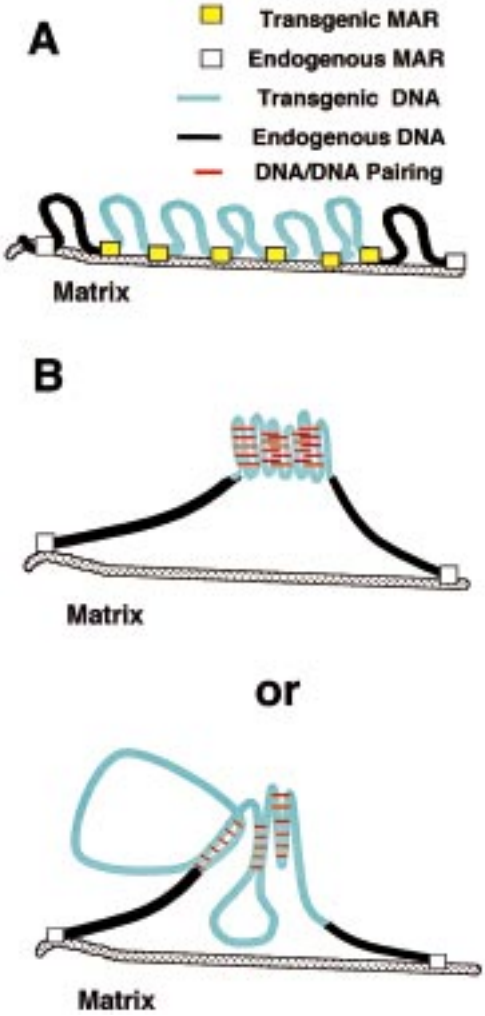

Figure 6. 'Carpet Tack' model to illustrate how flanking MARs might reduce repeat-induced gene silencing by physically limiting pairing interactions between adjacent copies of a transgene. A. The top portion of the figure represents a transgenic locus produced by integrating several copies of a DNA construct with flanking MAR elements (yellow boxes). The MAR elements are visualized as binding to the nuclear matrix (hatched region) to prevent pairing interactions between copies of the integrated transgene. B. In the absence of MAR elements, multiple transgenes (blue lines) may undergo various types of DNA/DNA pairing interactions that could lead to gene silencing. Only two examples of pairing interactions are shown in the schematic, but many other interactions can be envisioned in complex loci.

Molecular mechanisms of MAR effects

Nearly all reports describing MAR effects on gene expression have used MAR elements that are large and complex regions of DNA which may contain more than one functional element (Bonifer *et al.*, 1994; Phi-Van and Strätling, 1996), and it has so far been impossible to measure matrix binding *in vivo*. Therefore, it remains to be determined whether MAR effects on transgene expression are actually mediated by binding to the nuclear matrix or by some other feature of MAR elements. Models of MAR function therefore include several in which matrix binding *in vivo* is not considered essential.

MARs as terminators

MARs may be imagined to act by serving as strong terminators. It is known that poor transcription termination can significantly reduce gene expression levels, and there are several possibilities for how this might happen. Transcriptional interference may occur if RNA polymerase continues to transcribe into surrounding transcription units (Ingelbrecht *et al.*, 1989, 1991; Thompson and Myatt, 1997; Greger and Proudfoot, 1998). In addition, it is also possible that transcriptional read-through could produce transcripts of promoter sequences in multicopy loci. Recent evidence suggests that accumulation of promoter tran-

scripts in the nucleus may cause transcriptional gene silencing (Al-Kaff *et al*., 1998; Jones *et al*., 1999; Mette *et al*., 1999; Sampson *et al*., in preparation). If MARs enhance termination, they might be expected to alleviate these problems at least partially.

Poor termination might also result in aberrant RNA products that induce post-transcriptional gene silencing (Metzlaff *et al*., 1997; Stam *et al*., 1997). Although the Vaucheret *et al*. (1998) experiments mentioned above indicate that MARs do not protect against post-transcriptional silencing induced by another locus, the termination model suggests they might act *in cis* to prevent its induction at a MAR-containing locus.

The chromatin opening model

The chromatin opening model (Figure 5) was originally presented by the Laemmli laboratory (Käs *et al*., 1993). It has long been known that H1 histone binds preferentially to AT-rich DNA (Renz, 1975). Later work in Laemmli's group showed that H1 binds cooperatively to MAR elements to nucleate further assembly of H1 molecules and condensation of chromatin (Izaurralde *et al*., 1989; Käs *et al*., 1989). Käs *et al*. (1993) then found that the oligopeptide distamycin could selectively titrate H1 away from the MAR DNA, resulting in a redistribution of the H1. Distamycin treatment also resulted in an increase in chromatin accessibility as measured by susceptibility of the DNA to restriction enzymes or topoisomerase II. Thus H1 compacts the chromatin whereas distamycin acts to open it.

The non-histone chromatin proteins of the HMG I/Y class interact with DNA through the minor groove in a manner analogous to the interaction of distamycin with DNA (Bustin and Reeves, 1996). In addition, Käs *et al*. (1993) found that HMG I/Y displaced H1 histone from MAR DNA. HMG I/Y was also found to be enriched in H1-depleted chromatin (Zhao *et al*., 1993). Thus, these proteins, interacting with AT-rich MAR elements, became prime candidates to drive chromatin opening in the cell.

It has long been known that HMG I/Y proteins are in greater abundance in rapidly dividing tissue than they are in differentiated tissue in mammals (Johnson *et al*., 1990; John *et al*., 1995). As described above, Thompson *et al*. (1994) have shown that MAR effects are greatest in mouse embryo cells, where HMG I/Y levels are expected to be high, and we have found MARs have a much greater effect in undifferentiated plant cells in culture than they do in plants (Allen *et al*., 1993, 1996; Ülker *et al*., 1999). Both groups have speculated that the levels of HMG I/Y protein content may be important for these differences.

The DNA-binding domain of HMG I/Y, known as the AT hook, has a high affinity for MAR DNA. In an elegant study, Strick and Laemmli (1995) demonstrated the biological significance of the AT hook by synthesizing *m*ultiple *AT h*ook proteins ('MATH' proteins). A protein containing 20 AT-hook domains (MATH20) was found to alter chromosome assembly in *Xenopus* egg extracts (Strick and Laemmli, 1995; Hart and Laemmli, 1998). MATH20 also has an effect on position effect variegation (PEV) in *Drosophila melanogaster* (Girard *et al*., 1998). Using a tetracycline-regulated *trans*-activation system (Gossen and Bujard, 1992), MATH20 could be induced at various times in eye development. When MATH20 was present during early development, PEV of the white gene in the eye tissue was not observed, whereas when MATH20 was absent PEV occurred normally. Because silencing of the white gene in PEV is known to involve a condensed chromatin structure, Girard *et al*. (1998) concluded that the presence of MATH20 modifies chromatin structure to prevent chromatin condensation, presumably by the mechanism similar to that proposed for HMGI/Y in the chromatin opening model.

The Girard *et al*. work also showed the importance of developmental timing. If the MATH20 protein is expressed prior to 60 h after egg laying, no PEV occurs in the developed eye. However, when the MATH20 was expressed after 72 h, PEV could no longer be suppressed. Thus, the differentiation state of the *Drosophila* egg cell appears to be critical for MATH20 activity.

Improving or enhancing integration

Very little is known about how DNA is integrated when transformation occurs. However, it has long been known that MAR elements and bent DNA elements are often found at sites of illegitimate recombination in animals (Sperry *et al*., 1989; Milot *et al*., 1992) and yeast (Rattray and Symington, 1993). MARs have also been found in the DNA flanking transgene integration sites in *Arabidopsis* (Sawasaki *et al*., 1998) and rice (Takano *et al*., 1997), suggesting that they may somehow create hotspots for integration. It is also possible that flanking MARs on the transforming DNA may somehow facilitate the integration process. As we noted earlier, chromatin proteins such

as histone H1 and HMG I/Y bind preferentially to MAR sequences (Zhao *et al*., 1993), and one might imagine such proteins protecting the incoming DNA, stimulating the integration process, or even helping to 'target' transforming DNA to active regions of the genome.

Preventing pairing interactions

Most current data are consistent with a 'carpet tack' model whereby MARs act by preventing *cis* interactions between multiple transgenes. Table 1 shows that the largest MAR effects have been obtained in direct transformation experiments, in which one would expect a preponderance of complex, multicopy loci. It is now well established in both animal (Wolffe, 1997; Wolffe and Matzke, 1999) and plant systems (Assaad *et al*., 1993; Ye and Signer, 1996) that silencing is more likely to occur when multiple transgenes are integrated at a single locus. However, our data show that multicopy loci are more frequently expressed if the transgene constructs are flanked by MARs.

According to the model shown in Figure 6, multicopy loci would be susceptible to silencing induced by pairing between the transgenes. However, the presence of interspersed MAR elements could cause chromatin to bind to the nuclear matrix frequently enough to prevent the *cis* interactions we believe are necessary for gene silencing. Thus, by binding to the matrix MARs might 'tack' an array of transgenes in place well enough to prevent homology sensing and the induction of gene silencing.

The prevention of pairing model is complicated by the observation that when the transgene copy number increases above a critical level, presence of MARs can no longer prevent silencing (Allen *et al*., 1993, 1996). We feel that there are two main possibilities. The first possibility is that, because MAR elements are very closely linked in our multicopy loci, multicopy arrays may saturate the available MAR-binding sites in a local region of the nuclear matrix. This model predicts that some of the transgenes in a high-copy array would be unable to bind to the nuclear matrix, in spite of being flanked by MARs, and that pairing interactions among the unbound sequences would make such loci susceptible to pairing-mediated silencing similar to that seen at loci without MARs.

The second possibility is that transgenes flanked by MARs are transcriptionally active, making quantities of mRNA that increase with gene copy number up to a threshold level, but that silencing is induced post-transcriptionally once the level of transgene mRNA exceeds that threshold. This possibility is intriguing because, as noted above, some of the initial work with animal cells showed that flanking A elements allow transgene expression to increase up to very high copy numbers (Phi-Van *et al*., 1990; Stief *et al*., 1989). We have already discussed the possibility that the A element may function as more than just a MAR. However, it is also possible that the animal cells used in these experiments were naturally less active in post-transcriptional silencing than the plant cells we have studied. The recent discovery of suppressors of post-transcriptional silencing, such as P1HC-Pro (Anandalakshmi *et al*., 1998), should help clarify this question.

Summary and future prospects

Many basic questions remain to be answered about MARs and other DNA elements that influence chromatin structure. As noted above, it remains to be determined whether MARs (especially those in transgene constructs) actually bind to the nuclear matrix or act to open chromatin as predicted by the models. Many putative MAR-binding proteins are being isolated, but a real understanding of MAR/protein interactions will require the ability to document association of these proteins with MARs *in vivo*. Perhaps one of the greatest hopes for progress in this area is the development of fluorescent probes that allow one to visualize dynamic interactions in living cells without fixation or extraction. Work using GFP fusions to chromatin proteins or DNA-binding proteins (e.g. Misteli *et al*., 1997; Straight *et al*., 1997; Belmont and Straight, 1998; Kanda *et al*., 1998; Verschure *et al*., 1999) may pave the way for studies of MAR-protein interactions *in vivo*.

We are only beginning to understand the intricate complexity of regulation that occurs in the nucleus, and it is likely that MAR function is also more complex than is yet widely appreciated. Several reports (Fey and Penman, 1988; Bidwell *et al*., 1994; Krauss *et al*., 1997; Choi *et al*., 1998) indicate that the composition of the nuclear matrix is altered in cancerous cells and varies in different cell types. Nuclear matrices from different cell types might therefore be imagined to favor different MARs, or to bind the same MAR with varying affinities (Cook, 1989). We think it likely that different MARs will eventually be shown to have specialized functions in different cell types.

Finally, from an applied technology viewpoint, the use of MARs is likely to increase the predictability and stability of transgenic traits. It should be noted, however, that these studies are still in an early phase. The full potential of MAR technology will not be known until several different MARs have been tested in plant breeding programs spanning multiple generations.

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